Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

Identification of the cellular and molecular mechanism by which CD2AP, a regulator of neuronal intracellular transport contributes to the development of Alzheimer's disease.

Cláudio Silva Ferreira

Dissertação

Mestrado em Biologia Molecular e Genética

2014

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

Identification of the cellular and molecular mechanism by which CD2AP, a regulator of neuronal intracellular transport contributes to the development of Alzheimer's disease.

Cláudio Silva Ferreira

Dissertação orientada por:

Dra. Cláudia G. Almeida

Professora Dra. Margarida Telhada

Mestrado em Biologia Molecular e Genética

2014

Pela minha avó,

Que criou-me desde que nasci, ate me ser levada pela Doença de Alzheimer.

Pelo meu avô

Que ajuda-me mais do que eu alguma vez poderei retribuir.

Acknowledgements

Agradeço profundamente à Claudia, minha orientadora que aceitou-me no seu projecto e permtiu que eu fize-se parte da sua equipa fantástica. A sua ajuda foi imensuravel, espero não ter desapontado.

Agradeço ao Florent e ao Ricado pelos vossos ensinamentos, admiro a vossa experiencia e conhecimento, prometo trabalhar para chegar ao vosso nivel.

Maltinha do Lab ou que por ele passou, Tatiana, Andre, Angela, Andreia, Laura e Noelia, obrigado por tudo, pela boa disposição, pela ajuda em altura de maior aperto, ou pelos doces que partilhamos á hora do lanche. Encontramo-nos numa jantarada brevemente.

Um agradecimento especial á minha familia, que me ajudou nestes 2 ultimos anos, sem a vossa ajuda nada disto teria sido possivel.

Ao meu amor, é impossivel agradecer, foste o meu pilar o meu ar, não sei como te poderei alguma vez retribuir o teu apoio.

“Que negra sina ver-me assim,

Que sorte e vil degradante,

Ai que saudades eu sinto em mim,

Do meu viver de estudante”

Fado do Estudante

Resumo em língua Portuguesa da Dissertação *Identification of the cellular and molecular mechanism by which CD2AP, a regulator of neuronal intracellular transport contributes to the development of Alzheimer's disease.*

A doença de Alzheimer (AD) foi identificada em 1907 pelo Dr. Alois Alzheimer. Trata-se de uma doença neurodegenerativa bastante complexa, sendo a maior causa de demência a nível mundial. Após mais de 100 anos de investigação na AD os únicos tratamentos alcançados apenas conseguem retardar a doença, e melhorar a qualidade de vida do paciente, por mais algum tempo.

A neuropatologia é caracterizada pela presença no cérebro de placas de péptidos β-amilóide (Aβ) e aglomerados intraneuronais de formas hiperfosforiladas da proteína tau associada aos microtúbulos. Numa fase inícial da AD inicia-se a acumulação e oligomerização do péptido Aβ, tanto a nível extra como intracelular que causa disfunção das sinapses levando à perda de sinapses e neurónios. Os principais sinais e sintomas clínicos da AD são a perda de memória, de habilidades intelectuais, racionais e sociais gerando a perda de qualidade de vida do paciente, acabando por num estado avançado levar á perda total da autonomia e da própria identidade do paciente, até à morte.

O péptido Aβ é um péptido de 39 a 43 aminoácidos produto do processamento sequencial da proteína precursora amilóide (APP) pelas enzimas β-secretase (BACE1) e posteriormente γ-secretase (via de processamento amilodoigénica). O processamento do APP origina dois péptidos $A\beta_{40}$ ou $A\beta_{42,}$ sendo o último o responsável pelo efeito de toxicidade nos neurónios. A anormal acumulação de $A\beta_{42}$ nos neurónios resulta de um desequilíbrio entre os níveis de produção e degradação do $A\beta_{42}$. A produção do $A\beta_{42}$ via o processamento de APP está dependende do encontro do APP e seus derivados com as secretases específicas do seu processamento nos endossomas. Sendo a desregulação do tráfego neuronal para os endosomas uma das causas possíveis para o desenvolvimento da AD.

A AD está fortemente associada à idade sendo o envelhecimento celular o principal factor de risco para o seu desenvolvimento. A AD pode ser classificada em dois tipos, a AD de aparecimento precoce ou a AD de aparecimento tardio (também conhecida por tipo familiar ou esporádico respectivamente). Contudo, estão identificados outros factores de risco para a doença como factores genéticos; predisposição clínica, ou comportamentos de risco.

Estudos de associação genética com a AD em populações de diversas regiões do

globo, identificaram um conjunto de genes com forte associação à AD de aparecimento tardio. O polimorfismo rs9349407 do CD2AP foi um dos genes identificados como factor de risco para AD, $p = 8.78E^{-07}$, odds racio (OR) = 1.08, 95 % intervalo de confiança. *Cluster of Differentiation 2-Associated Protein* (CD2AP) é uma proteína citoplasmática reconhecida pela sua função em células T, podócitos, ou pela sua interacção com actina. É referida como sendo uma proteína efectora da Rab4 na maturação dos endossomas. A função desempenhada nos neurónios e no desenvolvimento da AD é completamente desconhecida.

O objectivo deste trabalho de dissertação é estudar o papel da proteína CD2AP nos mecanismos celulares e moleculares, que podem levar ao desenvolvimento da AD. A nossa hipótese biológica é que o CD2AP como regulador da via endocítica neuronal tem acção na acumulação de Aβ42 nos neurónios levando ao desenvolvimento da AD. Para estudar o CD2AP utilizamos uma linha celular de neuroblastoma de rato (N2a) e culturas primárias de neurónios (PN) de murganho (Balb-C). Iniciamos o estudo do CD2AP comprovando a expressão endógena do CD2AP nos neurónios primários e células N2a. Observamos uma distribuição homogénea do CD2AP no corpo celular e dendrites dos primários neurónios, e um enriquecimento junto á membrana plasmática e espiculas. Nos axónios a concentração de CD2AP era menor. Nas células N2a verificamos por imunofluorescência que o CD2AP se distribui por toda a célula com uma maior concentração na região perinuclear (PR). Para a subexpressão do CD2AP nas células utilizamos uma construção do CD2AP marcada por uma proteína de fluorescência verde (GFP-CD2AP), e uma segunda construção que possuí um codão stop após o segundo domínio SH3 (GFP-CD2AP(SH3)). Verificamos que o GFP-CD2AP nas células tem tendência a acumular-se junto á membrana plasmática e processos celulares, e que ao contrário da proteína endógena o GFP-CD2AP aglomera e forma grandes e brilhantes vesiculas. Verificamos também que os dois primeiros domínios do GFP-CD2AP(SH3) alteraram a dispersão do CD2AP pelas células.

Tendo em conta a hipotese colocada utilizámos a técnica de transfecção de DNA e RNA de interferência alteramos a expressão do CD2AP nas células e analisámos os níveis de Aβ42 em células a sobrexpressar o CD2AP ou tratadas com siRNA contra CD2AP (subexpressando CD2AP). Detectámos um aumento significativo dos níveis de Aβ42 nas células a sobrexpressar CD2AP, de igual forma se registou o aumento de Aβ42 nas células a subexpressar CD2AP. Este resultado sugere que para CD2AP funcionar normalmente os seus

níveis são regulados com precisão pelos neurónios sendo que excesso ou falta de CD2AP induz um aumento de Aβ42.

Seguidamente, investigámos se o CD2AP regula o processamento do APP em PN e em células N2a, em células a sobrexpressar ou a subexpressar o CD2AP. Comprovámos por western blot que o CD2AP tem tendência a diminuir o processamento do APP. Sugerindo que o CD2AP regula o processamento do APP pela γ-secretase, reacção enzimática que forma o péptido Aβ.

Por imunofluorescência analisámos o efeito do CD2AP na localização endossomal do APP e da BACE1 nas células N2a. Destacamos que em células N2a tratadas com siRNA-CD2AP ocorre um aumento do APP/CTFs transportado por endossomas positivos para Lamp1. Sugerimos que nas células que subexpressam CD2AP ocorre um aumento do encontro do APP/CTFs com a γ-secretase, levando ao aumento de produção do $A\beta_{40/42}$.

Porque o CD2AP esta descrito como tendo uma função reguladora da estrutura F-actina e que a F-actina participa na dinamica dos endosomas.Verificamos por imunofluorescência o efeito do CD2AP na regulação da F-actina nas células N2a e PN, comprovamos que as células tratadas com siRNA-CD2AP assumem uma morfologia differente com a presença de longas extensões da membrana plasmática, que poderam ser enormes filopodia ricas em F-actina, semelhantes às que se observam em zonas de crescimento de processos neuronais. Verificàmos também que os níveis de F-actina estão aumentados nas células que subexpressam siRNA-CD2AP.

Em suma, comprovamos que o CD2AP é uma proteína com os níveis de expressão altamente regulados pela célula, que uma alteração dos níveis de expressão do CD2AP provoca um aumento da acumulação do Aβ42 nas células, possivelmente associado ao efeito do CD2AP na maturação dos endossomas e na regulução do citosqueleto de actina.

Palavras-chave: CD2AP; Beta-Amilóide, Proteina precursora Amiloidogénica; Doença de Alzheimer, BACE1,

Abstract

CD2AP is a cytoplasmatic protein known to be an effector of maturation of endosomes. Studies of genetic associations recognized CD2AP as a risk factor of Alzheimer's disease. The intraneuronal accumulation of beta-amyloid 42 (Aβ42) peptide is a hallmark of early stage of Alzheimer's disease. In this dissertation we studied the effect of CD2AP on the accumulation of cell associated Aβ42, in primary mouse neuronal culture (PN) from cerebral cortices of BALB/c mice with 16 days of gestation and a cell line of mouse neuroblastoma cells (N2a).

Using cells, transiently transfected with CD2AP or treated with siRNA against CD2AP, we describe CD2AP effects on cells that may lead to development of Alzheimer's disease. We found that the deregulation of CD2AP expression level lead to increase of Aβ42 in the cells. The Aβ42 is product from sequential processing of the amyloid precursor protein (APP) by β-secretase enzyme (BACE1) and next γ-secretase. Cells overexpressing or depleted for CD2AP have tendency to decrease mainly the levels of the C-terminal fragments (CTFs) of APP. We described in cells depleted for CD2AP an increase of APP/CTFs in late endosomes, suggesting that an increase in the meeting of APP with the γ-secretase. We analyzed the BACE1 endocytosis pathway in cells overexpressing CD2AP, we did not find significant differences in the distribution of BACE1.

Knowing that CD2AP regulates actin polimerization, we studied the effect of CD2AP depletion in cells. CD2AP knockdown caused an increase in F-actin level and altered the morphology of cells.

I suggest that the level of expression of CD2AP is highly regulated in the cell, and that the interaction of CD2AP in the maturation of cells leads to increase on cell associated Aβ42. Justifying CD2AP as risk factor for the AD.

Key-words: CD2AP; Beta Amyloid, Amyloid Precursor Protein; Alzheimer's Disease; BACE1,

Index

Index of Figures and Tables

Abbreviations

APP	Amyloid Percursor Protein
Aβ	Beta amyloid
CCV	Clathrin-coated vesicle
CD2AP	Cluster of Differentiation 2-Associated Protein
CTF	C-Terminal Fragment
EE	Early Endosome
EOAD	Early-onset Alzheimer Disease
ER	Endoplasma Reticulum
GWAS	Genome Wide Association Study
IF	Immunofluorescence
KD	Knock-Down
LE	Late Endosomes
LOAD	Late-onset Alzheimer Disease
LY	Lysosome
MVB	Multivesicular Body
NFT	Neurofibrillary Tangle
PM	Plasma Membrane
PN	Primary Neurons
PR	Perinuclear Region
RE	Recycling Endosome
RT	Room Temperature
siRNA	Small Interfering RNA
TfR	Transferrin Receptor
TGN	Trans-Golgi Network
WB	Weston Blot
WT	Wild Type

Introduction

Alzheimer Disease

In 1907 the German physician Dr. Alois Alzheimer identified the first case of Alzheimer disease in a old-woman that he followed the last years of her life having observed a unique clinical-pathological process, set of symptoms, namely, progressive memory loss, confusion, disorientation and delusions ([1]). During almost five years, her mental capacities continued to deteriorate rapidly, until she finally died in a completely demented state. After patient's death, Alzheimer performed an autopsy on her brain, finding significant cerebral atrophy and the presence of unusual plaques and tangles ([1]). His characterization and notes on the case led to the discovery of Alzheimer's disease (AD).

Nowadays AD is the most common form of dementia, it is estimated that in 2030, 66 million people worldwide will be living with dementia ([2]) . It is recognized as a major public health problem in developed nations and the third more expensive disease to treat in the U.S., costing society close to $100 billion annually. There is currently no cure for AD and the treatments available to patients, only serve to slow down the inevitable progression of the disease and improve the quality of life of the patient.

This neuropathology is characterized by presence of plaques of amyloid β (Aβ) peptides and intraneuronal tangles of hyperphosphorylated forms of microtubule-associated protein tau. Prior to amyloid plaques, it is amyloid accumulation and oligomerization of beta-amyloid both extra and intracellular that causes synapse dysfunction eventually leading to loss of synapses and neurons. The principal signs are the loss of memory, intellectual, rational and social abilities leading to a loss of life quality.

Beta-amyloid accumulation causes AD

In patients with AD the progressive accumulation of Aβ, results from an imbalance between the levels of Aβ production, aggregation and degradation in the brain. The progressive accumulation of Ab occurs intracellularly. Indeed, Aβ42 enrichment in endosomes (multivesicular body or MVBs) near synapses is involved in the synapse dysfunction, the most established correlation with cognitive decline in AD ([3,4]). Eventually, neuronal processes filled with ab42 degenerate and spill out Ab42. This high concentration of Ab42 could seed the formation of amyloid plaques. Because, the secreted form of Aβ42 is

hydrophobic it potentially gradually increases in the extracellular space and to begin aggregating until it contributes to the formation of insoluble Aβ plaques. Aβ plaques spread their toxicity to surrounding neurons and their processes ([5;4]).

Additionally, to extracellular Aβ42 accumulation there is also an increase in Aβ42 intracellularly. This enrichment in multivesicular body (MVB) near synapses is involved in the synapse dysfunction, the most established correlation with cognitive decline in AD ([3,4]).

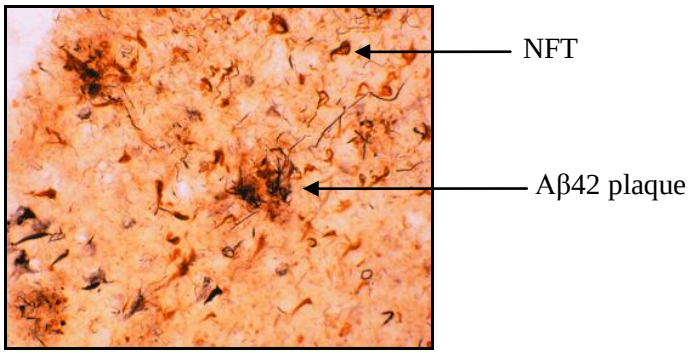


Figure I – Slide of tissue brain from AD patient. AD brain shows Aβ42 extracellular accumulation and amyloid plaques formation as well as neurofibrillary tangle from hyperphosphorylated tau protein. Image from Royal Society of Chemistry.

Many studies focus on the mechanism by which Aβ affects synapses but the answers are still unclear. It is now consensual that Aβ affects glutamate receptors localization and function at synapses, that has an important role in synaptic plasticity, a cellular mechanism involved in the process of formation of memory and learning ([6]).

Amyloid precursor protein trafficking and processing

Endocytosis is the eukaryotic process by which cells uptake fluids, solutes (pinocytosis) or macromolecules, even other cells (phagocytosis), by PM invagination. Endocytosis is a vital process for cell life and cell-to-cell, or cell-tissue communication. Actually we know four mechanisms of pinocytosis: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin/cavelolae-independent endocytosis ([7,8]). The most understood pathway is the clathrin-mediated endocytosis that I will refer to it only by endocytosis.

PM is rich in specific protein receptors that change depending on the cell necessities. Macromolecules and their receptors accumulate in clathrin-coated pits region, the clathrin-

coated pits, invaginate into the cell and pinch off to form clathrin-coated vesicle (CCVs). Clathrin is a trikelion (three-legged) structure formed by three clathrin heavy chain and each bound to clathrin light chain, and the heterotetramer assembly protein 2(AP2). AP2 bound directly or indirectly clathrin to the transmembrane receptors. CCVs uncoat and are able to fuse with EE, the CCVs have in the cytosolic domain receptors with sorting signals to regulate the pathway destination. After the fission of CCV's from PM they fuse with EE. The macromolecules in the EE, after 5-15 min move to LE/LY for degradation in the perinuclear region, this process is accompanied by the release of Rab5 and Rab7 binding. These Rabs are members of GTPase protein family that partly control the vesicular transport. From EE the cargo also can return to PM following the recycling pathway to begin again the endocytosis cycle and signaling. Cargo can also be sorted to the recycling endosomes (RE), from where when needed can recycle to the PM. ([27])

The cargo that does not get sorted stays in the endosome and may undergo multivesicular sorting. Multivesicular sorting of cargo occurs during endosomal maturation and consists of segregating receptors from the endosomal limiting membrane to the endosomal lumen by the formation of inner luminal vesicles, forming multivesicular bodies (MVBs). MVBs are LE that eventually fuse or deliver their contents to LYLE and LY are filled with soluble hydrolytic enzymes (proteases, glycosidases, lipases phosphatases and sulfatases) crucial to control the intracellular cargo degradation. To optimize enzymes efficiency, the lumen has acidic pH (~5). After macromolecules digestion the transport proteins in the LY membrane carry the final products to the cytosol, for reutilization. LY receive the lysosomal proteins from the lumen of ER, transported through the Golgi apparatus by means of CCV's ([8]).

The Aβ peptide is a 39-43 amino acid peptide derived from amyloid precursor protein (APP) by sequential proteolytic cleavage. APP is a transmembrane glycoprotein with a large extracellular/lumenal domain and a short cytoplasmic domain at C-terminal. It is expressed as three isoforms: APP695 (neuronal form), APP770 and APP751 (non-neuronal glial cells form) ([9]).

The processing of APP can occur by two pathways, non-amyloidogenic (does not produce Aβ) and amyloidogenic pathway. In amyloidogenic pathway APP is cleaved in plasmatic membrane (PM) or in early endosomes (EE) by β-secretase, producing a secreted

form of APP (sAPPβ) and membrane-bound C99. The C99 fragment is then cleaved in early endosomes (EE) or in late endosomes (LE) by γ-secretase to generate Aβ40 or Aβ42 ([10,9]). A small variation on APP cleavage site for β-Secretase could produce a shorter C-terminal C89 that will which originate truncated amyloid species $A\beta_{11\text{-}40/42}$ ([11,12]). The enzyme β-Secretase is a protein named BACE1 (β-site APP cleaving enzyme) that belongs to the aspartyl-proteases family. γ-Secretase is a hetero-oligomer formed at least by four protein components, presenilins (PS-1/PS-2), nicastrin, APH-1 and PEN-2 ([13]).

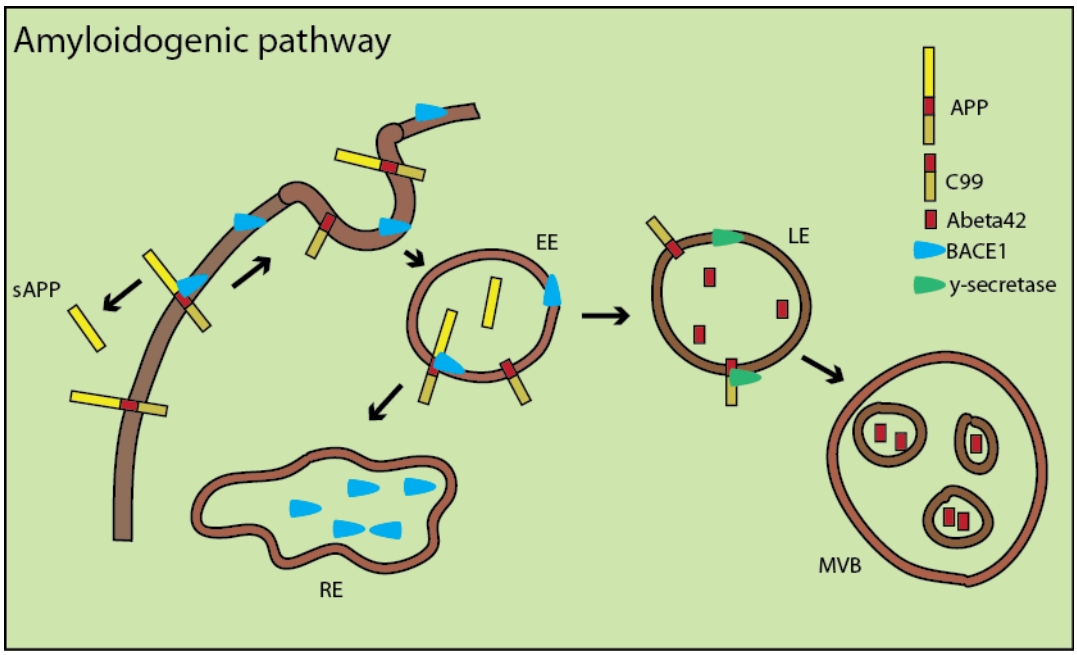


Figure II - APP processing pathways. Description of APP processing by amyloidogenic pathway, from membrane until multivesicular bodies

As other transmembrane proteins APP and BACE1 mature through the constitutive secretory pathway from endoplasmic reticulum (ER) to the PM. Higher concentration of APP reside in the Golgi and only a small part is transported for PM, of this only 50% is endocytosed by EE. On the EE one fraction of APP is recycled back to the PM and another fraction is targeted to the lysosome (LY) for degradation ([11]). Recent studies described that BACE1 on the cell surface is rapidly internalized, by the AP2-mediated clathrin-dependent pathway or by the clathrin-independent Arf6-dependent pathway, delivered in both situations to early endosomes (EE) ([14]) to and then sorted to recycling endosomes (RE) ([15]). From EE or RE BACE1 can recycle to the PM. Other studies have different suggestions for the pathways used, like BACE1 being recycled from EE to the TGN ([16, 17, 18]) or otherwise degraded ([19]) from EE to LE/LY. Overall the itineraries of BACE1 and APP are distinct except for the PM

and EE, since BACE is only active at acidic pH consensus now points to the EE as the site for Aβ production. Recent studies indicate that intracellular trafficking of APP and BACE1 plays an important role in Aβ production. In non-neuronal cells, Aβ production has been described to occur in multiple subcellular organelles, including the ER/ER-Golgi intermediate compartment, during its biosynthetic transport through Golgi, or in endosomes after endocytosis from the PM (Fig. II)([6;8; 20]). In neurons, the endosomes are now the most consensual organelle for Aβ production.

Understanding how APP, BACE1 and γ-secretase are trafficked through their pathways, and how the new identified risk factor genes associated with endocytosis, can interact on the different pathways will provide important insights that could help in the development of therapeutic drugs and strategies in the future for AD patients.

Late-Onset Alzheimer Disease

AD can be classified in two types, late-onset AD (LOAD) and early-onset AD (EOAD). EOAD also termed familial AD occurs before 65 years and is caused by rare mutations at *amyloid precursor protein* (*APP*), *presenilin 1* (*PSEN1*), and *presenilin 2* (*PSEN2*) genes involved with γ-secretase, resulting in Aβ42 increase ([21,22]). The prevalence of familial AD is below 5%. ([23])

LOAD also termed sporadic AD has as main risk factor the aging. Conjugated with genetic factors, environmental factors and lifestyle it doubles the risk of sporadic AD in double every five years after 65 years of age. LOAD has a estimated prevalence of 95% ([21]) without a known cause. Several epidemiological studies have shown that hypertension, atherosclerosis, coronary heart disease, smoking, obesity and diabetes are risk factors for sporadic AD. On the other hand, there is evidence that vitamins like B12, C and E or a moderate alcohol (specially wine) and caffeine intake have a protector effect ([22]) also like physical and mental exercise.

Until 2009, the genetic studies only identified the ε4-allele of the *apolipoprotein E gene (APOE)* as an established susceptibility marker for LOAD ([21]). In the last few years, several Genome-Wide Association Studies (GWAS) and meta-analysis have been published, where they identified a set of genetic risk factors for LOAD: *ABCA7, BIN1, CD2AP, CD33, CLU, CR1*, *EPHA1, MS4A6A*, *MS4A4E,* and *PICALM* ([24–26]). Additionally, recent studies have identified rare variants in the APP, and phospholipase D3 that also confer

protection or risk against LOAD. ([27]) These genes have a known role at inflammatory, lipid metabolism or endocytosis pathway, but the specific role in AD is still unknown. It is of utmost importance to identify the cellular mechanisms by which they are involved in AD.

CD2-associated protein

CD2AP, for cluster of differentiation 2-associated protein also known as Cas ligand with Multiple SH3 domains (CMS), is an 80kDa cytoplasmatic protein containing three SH3 domain (involved in protein-protein interactions) in its NH_2 terminus, a proline-rich region and a COOH- terminal coiled-coil domain ([28,29]). In Humans, *CD2AP* gene is located in chromosome 6 (6p12).

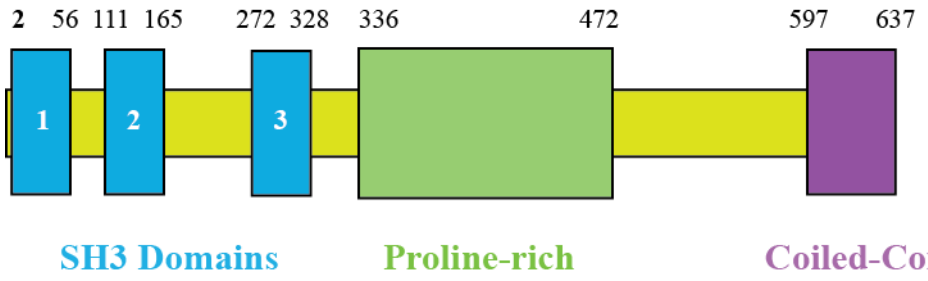


Figure III – CD2AP protein structure. Different functional domains of CD2AP: three Src homology 3 (SH3) domains at N-terminal; a proline-rich domain in the middle; and a coiled-coil region at C-terminal ([29]).

CD2AP was found for the first time as a ligand for T-cell adhesion protein CD2, were name came from ([30, 31]). In 2002 was published that CD2AP control T-cell activation, and the apoptosis in neuronal cells ([32]), interact with F-actin ([33]) . CD2AP is also known by the key role in podocyte homeostasis. CD2AP haploinsufficiency or a CD2AP SNPs are cause for renal failure. CD2AP serves as a linker anchoring for slit diaphragm proteins, podocin and nephrin, to the actin cytoskeleton of podocytes ([34]). In 2003, M. Cormont ([29]) suggested that CD2AP through its interactions with c-Cbl and Rab4, control early endosome morphology, and also that CD2AP could act as a Rab4 effector involved in sorting from EE to LE. Additionally, she proved that Horseradish peroxidase uptake was modified by CD2AP overexpression at late time points and the morphology of Rab7 positive endosomes was affected by the coexpression of Rab4 and CD2AP. In other study, it was described that CD2AP plays a role in cytokinesis, and was involved in the latest step, of cells scission, and the first two SH3 domains were crucial for interaction of CD2AP with anillin ([30]). In 2007

Gauthier et al., show in HeLa cells that CD2AP act as protein adaptor between endosomes and actin cytoskeleton ([35]).

As previously mentioned, in last four years the GWAS identified several risk factors genes, being made a list of the most significantly associated genes. From Alzheimer's Disease Genetics Consortium (ADGC) GWAS, CD2AP has an association with AD ($1\times10^{-6}\geq p>5\times10^{-8}$) ([24,25]) GWAS show that the association with AD is made by CD2AP SNP and not by the pattern of expression. Using meta-analysis, was described in 2014 a significant association between the CD2AP rs9349407 polymorphism and AD with $p = 8.78\text{x}10^{-07}$, odds ratio (OR) = 1.08, 95 % confidence interval. This association was proved for East Asian, American, Canadian, and European populations ([36]). Until now, limited information exists on the mechanism by which CD2AP interfere or interact in neurons and consequently in AD, the studies about CD2AP and AD refer that CD2AP behaves similarly to CIN85 the homologs of which have been identified as suppressors of Aβ toxicity in yeast and *C. elegans* ([37]). Another study in Drosophila found that loss of the fly ortholog for CD2AP and CIN85, increased tau neurotoxicity in tau-transgenic flies, further suggesting CD2AP normally functions in a protective role against AD risk ([38]) or in synaptic dysfunction and cell membrane processes ([39]) . None of these studies mentioned, presents evidence for a role of CD2AP in neurons.

It is of utmost importance to know the mechanisms by which CD2AP interfere with AD, to be possible develop new therapies and treatments.

Methods

Mouse Primary Neuronal Cultures

Primary mouse neuronal culture (PN) was prepared from cerebral cortices of BALB/c mice with 16 days of gestation (E16). BALB/c mice were homozygous wild type.

E16 cortices were dissected under magnifying glass in ice-cold Hanks balanced salt solution (HBSS) (Gibco, Carlsbad CA) with 0.45% glucose (from 50% D-glucose stock in water) and HEPES at 10mM (Gibco, [1 M]) pre-filtered. The cortical tissue was digested with trypsin (Lifetechnologies, Trypsin-EDTA (1X)) for 15min at 37ºC. Trypsin activity was stopped by washing three times with HBSS/Glucose and one Dubelco Modified Eagle's medium (Gibco, DMEM High glucose) with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Gibco, Penicillin-Streptomycin, Liquid) were performed. Neurons were dissociated with a glass pipete and plated on poly-D-lysine (100 µg/ml, SIGMA) coated plates) ($6x10^5$ cells/cm^2) and glass coverslips (cells/cm^2). Cultures were maintained in incubator at 7% CO_2/93% air. After 1 day *in vitro* (1 DIV) the medium was substituted for neurobasal medium (Gibco, Neurobasal Medium (1X)) with 2% B27 serum-free supplement (Gibco, B-27 Supplement, custom (50X)), 2 mM glutax (Gibco, Glutamine (100X)) and 1% Penicillin/Streptomycin.

Primary neuronal cultures were used with 9-12 DIV, but they were viable for 3-4 weeks under these culture conditions. The preparation of PN was shared by all lab members. I participated occasionally in the coating with poly-D-lysine and planting the neurons.

Cell Lines

The mouse neuroblastoma cell line, are used as cellular model of neurons, they share several properties with neurons and has been used in several studies on the AD ([40]) . Cultures were maintained in incubator at 5% CO_2/95% air, plated in 10 cm dishes with 10ml medium, 90% DMEM (Gibco, DMEM with Glutamax) , 10% FBS (Sigma, Fetal Bovine Serum, Research Grade) and 1:2000 Penicillin/Streptomycin. The N2a used in IF were plated on coverslips ($6x10^5$ cells/cm^2) and N2a for WB were plated on 6-well plate ($6x10^5$ cells/cm^2).

Plasmids

CD2AP plasmids were kindly provided by Mireille Cormont PhD from Faculty of Medicine, University of Nice. Truncated forms of CD2AP were obtained by introduction of stop mutations at 175 position, by using site-directed mutagenesis ([30]). The APP-mRFP construct is a kind gift from Stefan Kins from University of Kaiserslautern ([41]). BACE1-GFP plasmid was obtained from Jean Baptiste Brault and Stephanie Miserey Lenkei (Institut Curie, Paris, France). The plasmids were amplified in competent *E. coli* by the method of heat shock, 0,5µl plasmid were mixed with 100 ml *E. coli* in one *Eppendorf* for 30 min on ice then *Eppendorf* were warmed in the bath for 20 sec at 42º C, followed by 2 min on ice. To *Eppendorf* were added 100µl LB Broth (Sigma) and a antibiotic corresponding to the plasmid resistance, bacteria were incubated on shaker for 1h at 37ºC. After the selection of bacteria transformed with the plasmid of interest, increases the production scale of E. coli until 200-250ml. To extract the plasmid we used a midi-prep kit (NZYtech). Correct sequence of plasmids was confirmed by sequencing.

Cells Transfections

Cells plated in 26/6-well plate, were transfected with mix solution with 0.5/2.5µg cDNA and 0,5/2,5µl Lipofectamine 2000 (Invitrogen) in 25µl Opti-Mem solution(Gibco), this solution were incubated previously for 20 min at RT. For transfections CD2AP KD we use siRNA-CD2AP at 10-25-50nM and 0.8µl Lipofectamine RNAiMax (Invitrogen) in 50µl Opti-Mem solution.

Cells were fixed for IF analyze or collected for WB at 24h or 72h incubation to cDNA transfection or siRNA transfection respectively.

Immunofluorescence

Primary neurons and cell lines were used 24h or 72h after DNA transfection or siRNA transfection respectively. After washed with PBS cells line were fixed for 20 min with 4% formaldehyde diluted in PBS at RT and PN with 4% formaldehyde + sucrose 4% diluted in PBS at RT. After fixation cells were washed 3 times with PBS. Cells were blocked for 1h at RT with FBS 2% BSA 1% in PBS. After blocking, cells were permeabilized with 0.1% saponin (Sigma) in PBS 60 min at RT for vesicular proteins stainings or in 0.3% Triton-X100 (Acros Organics) 5 min at RT for cytoskeletal proteins stainings. Cells were imnunolabeled with primary antibody diluted in block solution, and incubated for 1h at RT, than washed four

times per 5min. After, cells were incubated with secondary antibodies in block solution for 1h at RT.

Primary antibodies	IF dilution	WB dilution	Supplier
Abeta42	1:50		Genetex
AnkyrinG	1:100	-	Santa Cruz
Anti-Green Fluorescente Protein	-	1:2500	Gift M.Arpin (Institute Curie)
APP 22C11	1:200	-	Millipore
APP P2-1	1:500	-	Invitrogen
APP Y188	1:200	1:1000	Genetex
CD2AP H-290	1:100	1:1000	Santa Cruz
EEA1	1:50	-	Santa Cruz
GM130	1:500	-	BD biosciences
Lamp-1/CD10	1:500	-	Santa Cruz
Myc	1:500	1:2500	Curie
Phalloidin	1:200	-	Invitrogen
TfR (h68)	1:50	-	Curie
α-tubulin	-	1:5000	Millipore
Secondary antibodies			
Alexa-488 anti-mouse	1/500	-	Molecular probes/Invitrogen
Alexa-488 anti-rabbit	1/500	-	Molecular probes/Invitrogen
Alexa-555 anti-Goat	1/500	-	Molecular probes/Invitrogen
Alexa-555 anti-mouse	1/500	-	Molecular probes/Invitrogen
Alexa-555 anti-rabbit	1/500	-	Molecular probes/Invitrogen
Alexa-647 anti-mouse	1/500	-	Molecular probes/Invitrogen
Alexa-647 anti-rabbit	1/500	-	Molecular probes/Invitrogen
HRP anti-mouse	-	1:5000	Bio-Rad
HRP anti-rabbit	-	1:5000	Bio-Rad
Probes			
Alexa Fluor™ 647-Conjugated Human Transferrin (1 mg/ml)	-	-	Jackson Immunoresearch Lab

Table 1 – List of antibodies and probes used in this study, dilutions and suppliers

Protein chemistry

Cells extracts

Primary neuronal and cell lines cultures plates, 24h after DNA transfection or 72h after siRNA transfection, were placed on ice and were washed with ice-cold PBS (phosphate buffered saline). Cells were lysed by scraping the plate with a rubber policeman after adding 100µl RIPA buffer (50nM Tris-HCl pH 7.4 (Sigma-Aldrich™), 1% NP-40 (Sigma-Aldrich™), 0.25% sodium deoxycholate (Sigma-Aldrich™), 150mM NaCl (NZYTech™), 1mM EGTA (Sigma-

Aldrich™) supplemented with protease inhibitor cocktail (Roche Diagnostics) Lysates were centrifuged for 15 min at 12000 x g at 4ºC. The supernatant was analysed by WB or snap frozen and stored at -80ºC until used.

Samples were separated using 10% or 15% Acrylamide SDS-PAGE gel, electrophoretic transfer to nitrocellulose membranes were performed at 40V for 1h. The membrane was blocked by incubation for 1 h with PBS containing 0.1% (v/v) Tween-20 and 5% (w/v) dried milk powder. Antibody incubations were performed in PBS Tween containing 2% (v/v) bovine serum albumin for 1h at RT, after 3 washes for 5 min each, to remove non-specifically bound primary antibody, membranes were incubated with secondary antibody diluted in PBST and 5% dried milk powder. After new round of washes the membranes were incubated with chemiluminescent substrate (GE Healthcare, ECL Prime WB Detection Reagent). The membranes were revel at Chemidoc XRS$^+$ (BioRad). Analysis of protein band intensities was performed using ImageJ 1.48g (NIH) software. To protein intensity signal was removed background signal. The band intensities of the proteins were then normalized to the corresponding α-tubulin band intensity.

Microscopy

Images were acquired on a Leica DMRA2 upright microscope, equipped with a CoolSNAP HQ CCD camera, using the 63x and 100x 1.4NA Oil immersion objective, DAPI + CY5 fluorescence filter sets and DIC optics, controlled with the MetaMorph V7.5.1/software. Images were analyzed with the software ImageJ 1.48g (NIH). The background signal was removed to fluorointensity signal.

Statistical analysis

Statistical comparisons of all data were made using t-test. The statistical t-test was made in Excel 2010 (Microsoft Office 2010). Anova was calculated in the site *http://vassarstats.net* .

Results

CD2AP expression and localization in neurons

According to genetic studies CD2AP is a risk factor for AD. Therefore, was indispensable to confirm the endogenous expression of CD2AP in neurons before starting the study of the effects of CD2AP on AD development. We compared the previously described expression of endogenous CD2AP in HeLa cells with the expression of CD2AP in a neuroblastoma cell line (N2a) and in primary neurons (PN) by western blot (WB) using specific CD2AP antibody (H-290) (Fig 1. A-C). Additionally, we expressed the GFP-CD2AP in N2A (Fig.1 C). In all western blots we observed a band above the 75kDa molecular weight marker, consistent with the expected size for CD2AP around 80kDa ([29]). In all WBs for CD2AP other bands appeared indicating the H-290 antibody is not as specific as previously published. Thus we confirmed if the antibody detected the exogenous expression GFP-CD2AP in n2a cells, indeed a band above 100kDa was detected consistent with the 80kDa size of CD2AP and with the 27kDa size of GFP. In PN a band of about 100 kDa consistently appeared and due to its strong intensity could be specific although it has not been previously described. The WB data indicates that CD2AP is expressed in neurons validating our hypothesis that CD2AP may function in neurons to contribute to the development of AD.

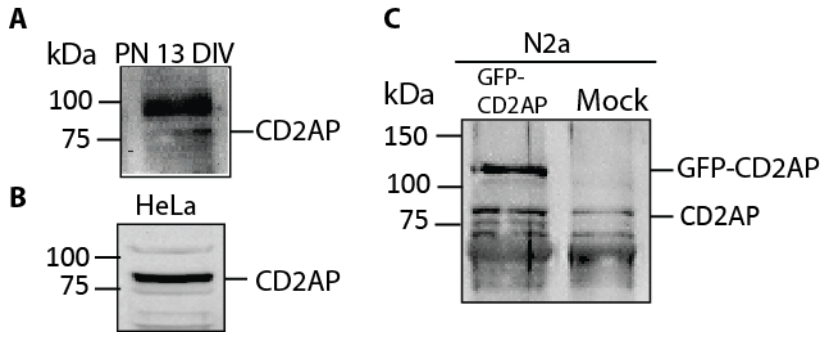


Figure 1 – Expression of CD2AP in primary neurons, HeLa and N2a Cells. WB analysis of endogenous CD2AP in RIPA-buffer extracted lysates from (**A**) PN (13 DIV) and (**B**) HeLa cells. (**C**) WB analysis of RIPA-buffer extracted lysates from N2a cells transiently transfected with GFP-CD2AP or not (mock). Anti-CD2AP antibody was used for all.

To better understand CD2AP was necessary analyze the cellular distribution of CD2AP in N2a, HeLa and PN cells. For that we immunolabeled N2a, HeLa and PN cells with anti-CD2AP to determine the cellular distribution of CD2AP (Fig. 2 A-C). CD2AP in PN was homogenously distributed in cell body and dendrites but less present in axons. In HeLa and N2a cells CD2AP spread for throughout the cell with a concentration in the perinuclear region

(PR) in some N2a cells (Fig. 2A), and an increase in the region of contact between two cells in HeLa cells consistent with CD2AP localization to the midbody (Fig 2.B) ([30]).

To study the effect of overexpressing CD2AP in Aβ accumulation, we used two constructs, GFP-tagged CD2AP and GFP-CD2AP(SH3) that has a stop codon after the second SH3 domain, the latter was used to determine the importance of CD2AP SH3 domains (Fig. 2D). We started by analyzing their distribution in N2a cells, HeLa and PN. Cells were transfected with GFP-CD2AP and GFP-CD2AP(SH3) (Fig.2 E-G). GFP-CD2AP localized to the plasma membrane, in filopodia and spines, and in the PR (Fig 2.E-G). GFP-CD2AP also had the tendency to aggregate and fill large and bright vesicles in the PR, this was more evident in HeLa cells. GFP-CD2AP(SH3) distribution was more diffuse and spread throughout the cells enrichment in the PM and spines, without forming aggregates evidenced a higher concentration in the PR (Fig 2.H-J). We conclude that the SH3 domains are not sufficient for the normal localization of CD2AP.

To study the effect of CD2AP knockdown on ab accumulation and to validate the results obtained on CD2AP expression on PN and N2a were transiently transfected PN and N2a with siRNA against CD2AP (siRNA-CD2AP). The siRNA-CD2AP sequence used was previously validated by Monzo et al., 2005 ([30]). We transiently transfected N2a with siRNA-CD2AP at 10nM and 25nM or with non-targeting siRNA (siRNA-control) (Fig 3.A, C). WB results exhibited a similar CD2AP decrease of nearly 90% at 10nM and 25nM. Therefore we chose to use the 10nM of siRNA-CD2AP for the following experiments. Next we analyzed CD2AP knockdown (KD) in PN (Fig 3.B, C.). We found that CD2AP expression decreased by 70%, a quite high KD considering that transfection of PN is not 100% efficient. Further, this assay also confirmed that the band above the 75kDa molecular weight marker corresponds to CD2AP. We also analyzed by immunofluorescence (IF) the depletion of CD2AP upon treatment with siRNA-CD2AP in N2A and PN and detected a decrease of CD2AP levels of 30% in N2a and 70% in PN (Fig. S1).

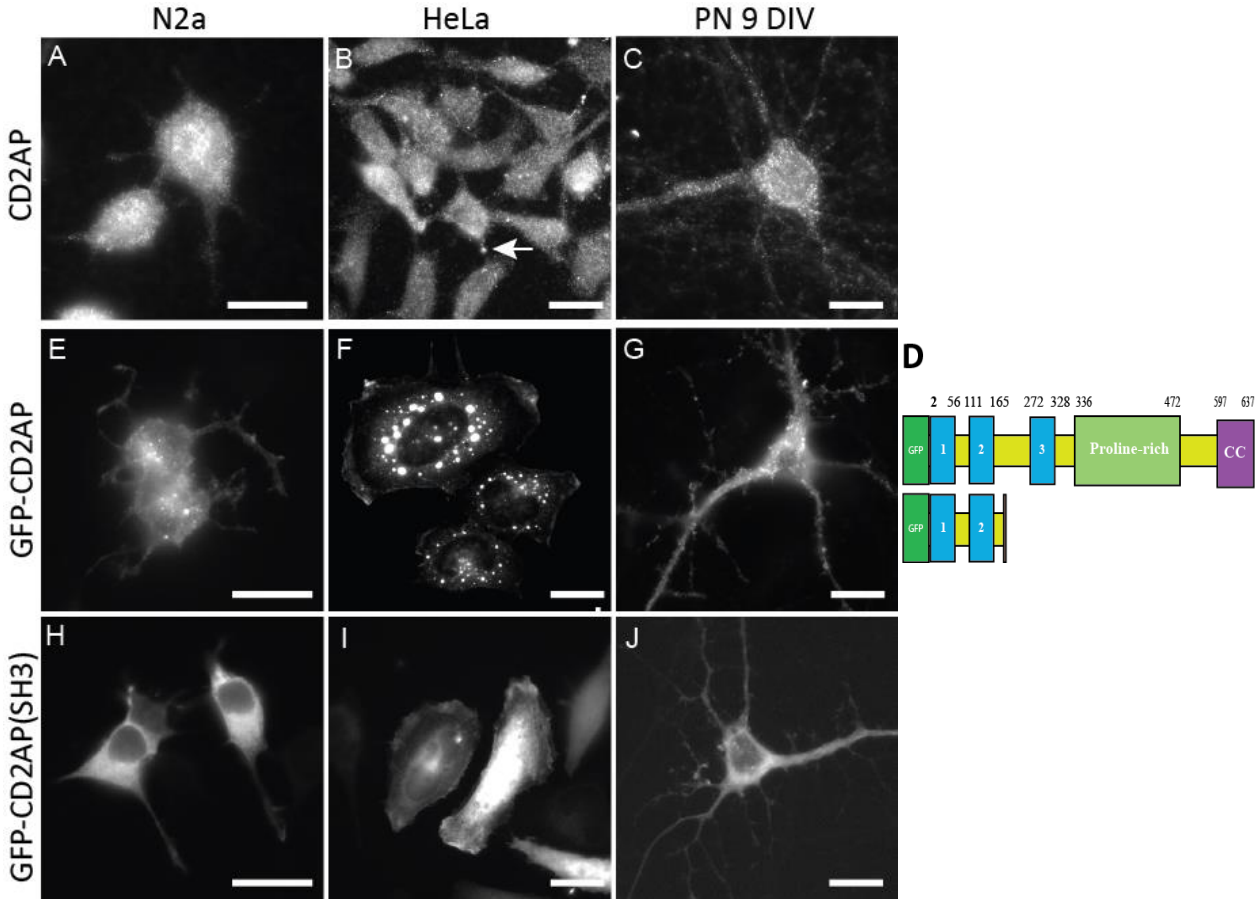


Figure 2 – CD2AP distribution in N2a, HeLa cells and primary neurons.

(A-C) Staining of the N2a, HeLa cells and PN with CD2AP antibody (H-290). (D) Plasmids encoding for GFP tagged CD2AP and GFP tagged CD2AP (SH3). (E-H) Cells transfected with CD2AP tagged with GFP. (I-G) Cells transfected with construct of CD2AP(SH3) tagged with GFP. Scale bar 20µm.

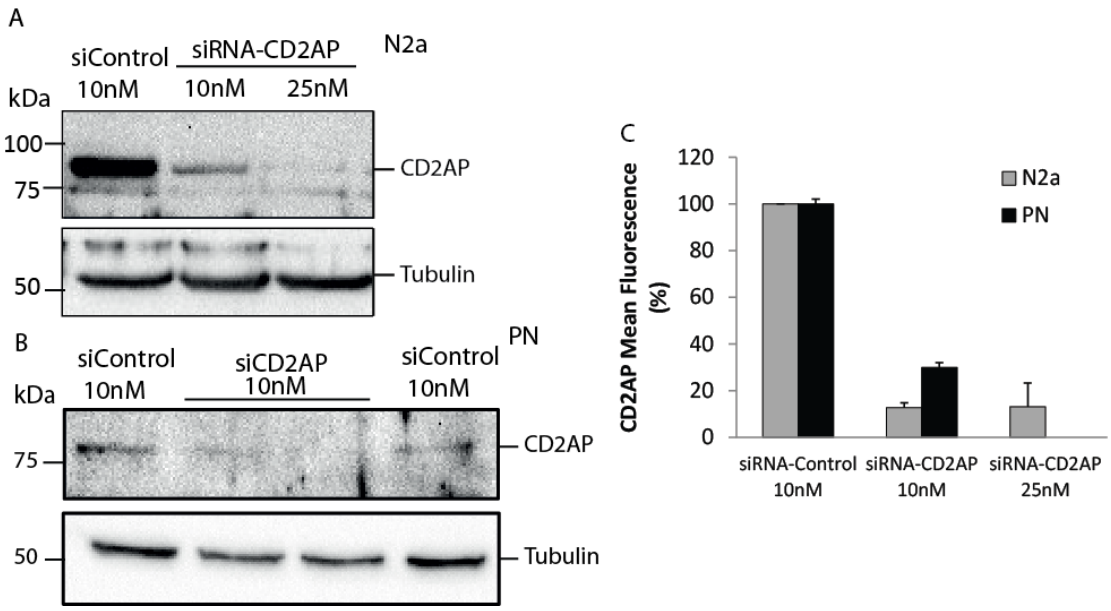


Figure 3 – CD2AP knockdown by siRNA treatment in primary neurons and N2a cells.

Cells were transfected with siRNA-CD2AP or siRNA-control at 10nM, using RNAiMax as described in Methods. Western blot analysis of N2a cells (A) and PN (13 DIV) (B) with antibodies against CD2AP and α-tubulin. (C) Densitometric analysis of WB of N2a and PN, normalized by total α-tubulin. N2a cells treated with siRNA-CD2AP reveal a CD2AP level of 12.7±2.1% at 10 nM (n=2) and of 13.1±10.14% at 25 nM (n=2). PN treated with siRNA-CD2AP at 10 nM (n=2) reveal a CD2AP level of 29.9±21.45%. The levels of CD2AP are shown as percentage of the levels measured in siRNA-control treated cells.

Effect of CD2AP on Abeta42 level

As the *CD2AP* is considered a risk factor for AD and the Aβ accumulation is involved in early stages of AD pathogenesis, we hypothesized that CD2AP may control the accumulation of Aβ. It is not known whether the CD2AP gene variant alters the levels of CD2AP in the brain or in neurons. Thus to test our hypothesis we decided to alter CD2AP expression in N2a cells and PN either by overexpression or by knockdown of CD2AP.

First, we investigated the effect of overexpression of CD2AP on abeta42 accumulation by transfecting PN and N2a cells with GFP and GFP-CD2AP. The effect of CD2AP overexpression in cell associated Aβ42 was analyzed by IF of PN and N2a cells anti-Abeta42 antibody (Fig 4.A-J). Aβ42 level was found to increase by 52% in GFP-CD2AP-expressing cells compared with GFP-expressing cells (Fig 4.K). In PN, Aβ42 level was analyzed in the cell body, dendrites and axons, to evaluate the differential accumulation of ab42 in these compartments. PN overexpressing CD2AP revealed its most important increase of 49% in dendrites followed by 32% in cell body. In axons ab42 accumulation was less evident with an increase of 21% GFP-CD2AP-expressing cells compared with GFP-expressing cells (Fig 4.L).

Having verified that CD2AP overexpression increases the Aβ42 level, we wondered if CD2AP KD also altered Aβ42 in the cell models. To start to test this hypothesis in N2a cells, cells were transfected with siRNA-Control and siRNA-CD2AP at 10 nM. After 72h of treatment cells were immunolabeled with anti-Abeta42 antibody (Fig 5.A-D). Aβ42 signal increased by 67% in N2a cells treated with siRNA-CD2AP (Fig 5.E). In PN Aβ42 signal increased by 74% in cell body and 49% in dendrites upon treatment with siRNA-CD2AP compared with PN treated with siRNA-control (Fig 5.F). These data was very interesting because they show an increase in Aβ level even when CD2AP is depleted. The fact that the depletion of CD2AP has the same impact as excess of CD2AP on ab42 accumulation suggests

that when overexpressed CD2AP function is compromised. This result suggests that the levels CD2AP necessary for its normal function are highly regulated by the cell. Overall, the deregulation of CD2AP expression leads to an increase of Abeta42, landmark of AD early stage, confirming our hypothesis.

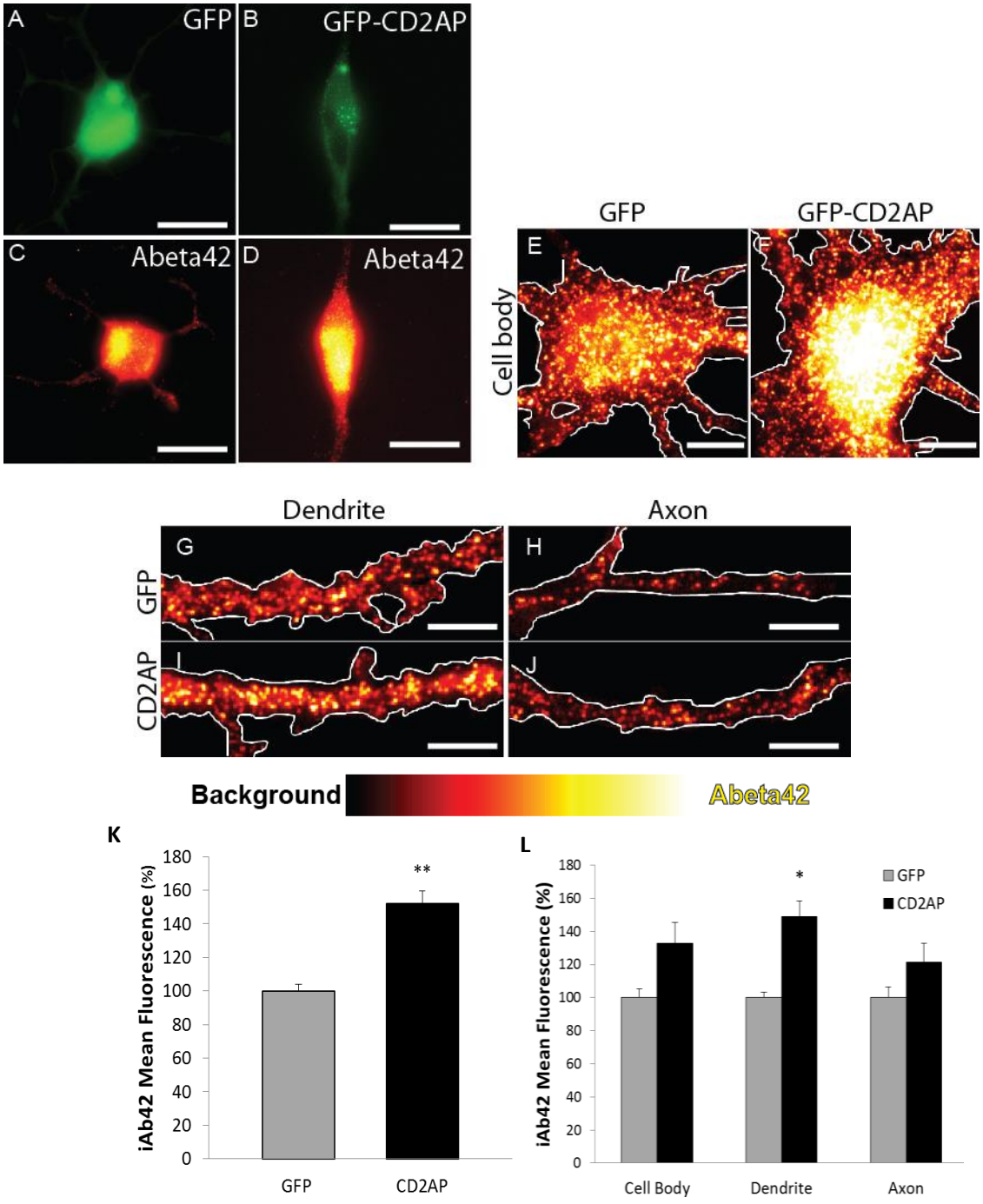


Figure 4 – CD2AP overexpression increases Aβ42 especially in dendrites.

Cells were transiently transfected, as described in *Methods*, with GFP-CD2AP and GFP, as control. Representative images of N2a cells expressing GFP (**A**) and GFP-CD2AP (**B**). Quantification of Aβ42

mean fluorescence in GFP (**C**) and GFP-CD2AP (**D**) expressing N2a cells. Representative images of (**E, F**) cell body, (**G, I**) dendrites and axons (**H, J**) from PN expressing GFP (**E, G, H**) and GFP-CD2AP (**F, I, J**). (**K**) Quantification of Aβ42 mean fluorescence in GFP- and GFP-CD2AP expressing cells. Abeta42 levels were increased in N2a cells expressing CD2AP (152.3 ± 7.3%, $p < 0.001$; n=3; N=70 cells) compared to GFP (n=3; N=72 cells). (**L**) Quantification of Aβ42 mean fluorescence in cell bodies, dendrites and axons of GFP and GFP-CD2AP expressing PN (11 DIV). Abeta42 levels were increased in cell bodies (132.7± 12.6%; n=3; N=23 cell bodies), dendrites (149.0 ± 9.3%; $p < 0.001$; n=3; N=61 dendrites) and axon (121.3 ± 11.6%; n=3; N=27 axons) of PN expressing CD2AP compared with GFP expressing PN. Scale bar 20µm.

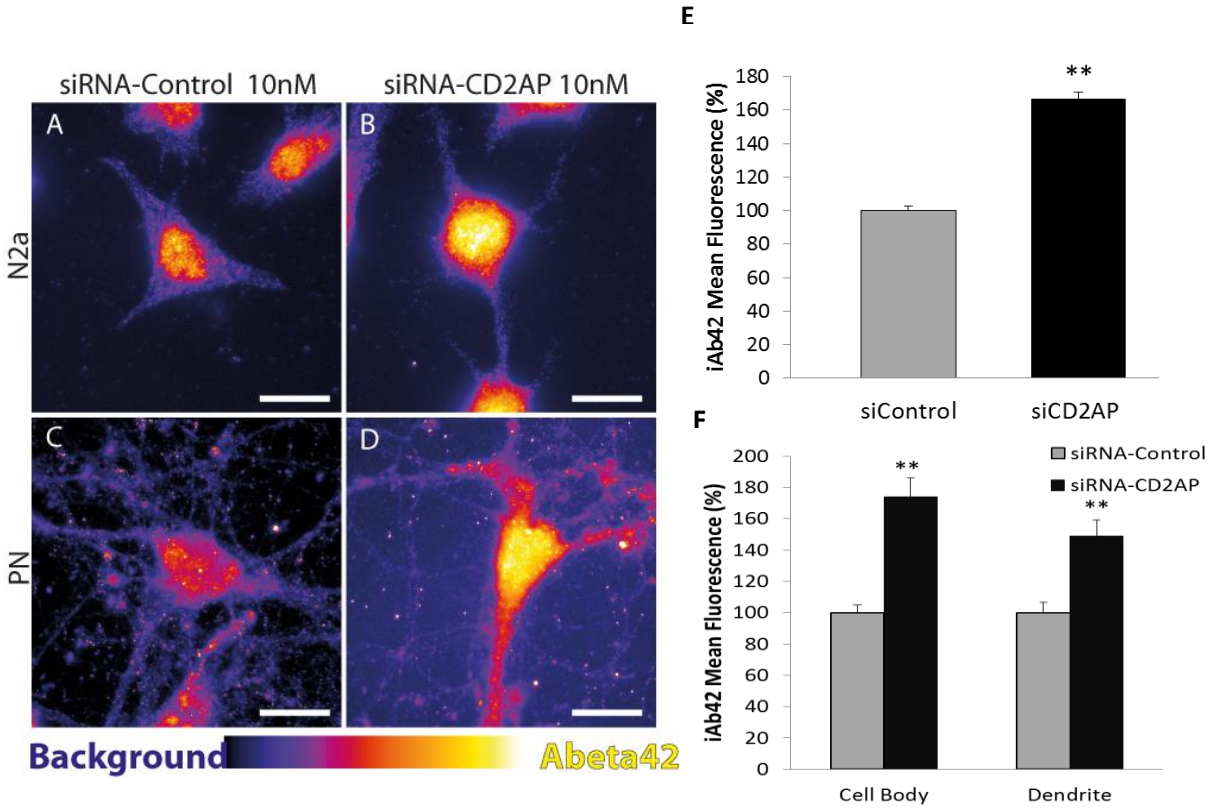


Figure 5 – CD2AP knockdown increases Aβ42 level.

N2a cells and PN were transfected with CD2AP-siRNA or siRNA-control, using RNAiMax as described in *Methods*. **(A-B/C-D)** N2a and PN cells where immunolabeled with anti-Aβ42, fluorescence intensity is pseudocolored in a blue-hot lut (dark blue is background, yeallow corresponds to the brightest signal)**. (E)** Quantification of mean Aβ42 fluorescence in N2a cells treated with CD2AP siRNA was 166.6 ±4,2 compared to N2a cells treated with siRNA-control ($p < 0.001 \times 10^{-36}$; N=208 CD2AP siRNA cells; N= 176 SiRNA Control cells; n=4). **(F)** Quantification of mean Aβ42 fluorescence in PN treated with CD2AP siRNA was 174.0 ±12,9% for cell body and 149,0 ± 10,2% for dendrites compared to PN treated with siRNA-control (p <0.001; N=35 CB; N= 47 DD, CD2AP siRNA cells; N= 35 CB; N=60 DD SiRNA Control cells; n=3). Scale bar 20µm.

Effect of CD2AP on APP Processing

The Aβ accumulation that we observed might be due to production excess or decreased degradation of Aβ. Since Aβ is the product of the APP processing, we next analyzed the

processing of APP through WB with anti-APP(Y188) upon overexpression of CD2AP in PN (Fig. 6.A). In parallel we overexpressed BACE1 as positive control since it increases APP processing. We determined the expression of GFP, GFP-CD2AP and GFP-BACE1 by WB with anti-GFP antibody (Fig S2). In all assays unexpectedly we could not detect GFP-BACE1 expression, although we always observed a notorious increase in APP processing, indicated by higher levels of APP CTF and of the ratio APP CTF over APP. CD2AP ovexpression slightly reduced APP levels by 13% and APP CTF's levels (C83/C99) by 25%. To analyze the amount of APP processed by the first cleavage we calculated the ratio CTF's/APP, then we notice that CD2AP overexpression decreases slighltly by 13% the ratio of CTF's/APP (Fig 6.B). In order to assess the effect of CD2AP KD on APP processing, we conducted WB analysis with anti-APPY188 of with PN treated with siRNA-CD2AP and siRNA-control at 10nM (Fig 7.A). CD2AP depletion induced a loss of total APP and a loss of 31% in the level of APP CTF's compared with siRNA-control treated PN (Fig 7.B). The rate of APP processing was reduced by 24% in PN treated with siRNA-CD2AP as compared to PN expressing CD2AP (siRNA-control). In summary the results of CD2AP overexpression or KD both point in the same direction, a small decrease of total APP and a significant decrease in APP/CTFs, suggesting that loss of function of CD2AP accelerates APP processing by γ-secretase leading to increased Aβ42 generation.

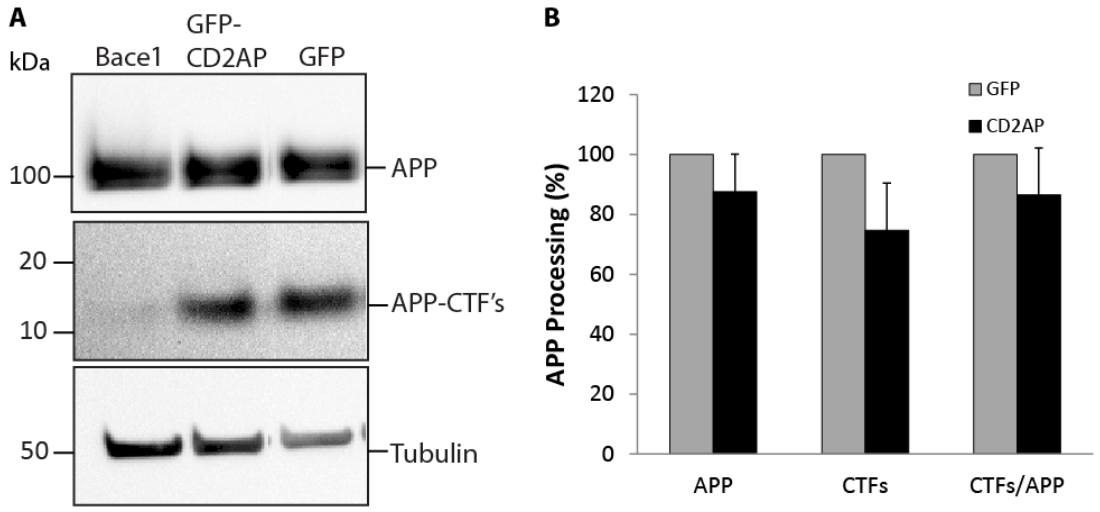


Figure 6 – CD2AP overexpression affects APP processing in primary neurons.

Primary neurons were transfected, as described in *Methods,* with GFP-CD2AP, BACE1-GFP positive control and GFP. **(A),** Western blot analysis of PN with anti-APP (Y188) and α-tubulin **(B).** Note that BACE-1-GFP expression in A. was undetectable. Note that in B. APP Y188 antibodies detects APP full length and APP CTFs (CTF C83 and C99) that are detected as one band. Densitometric analysis of WB of PN, normalized by total α-tubulin. PN expressing GFP-CD2AP reveal APP level of 87.6% and APP CTFs of 74.7% compared to GFP-expressing PN. The ratio of CTFs/APP was 86.5% compared

with GFP-expressing PN. The results are shown as percentage of the levels measured in GFP-expressing PN (n=3) (**C**). Note the analysis of APP levels upon BACE1-GFP expression was excluded because signal did not appear in western labeled for GFP.

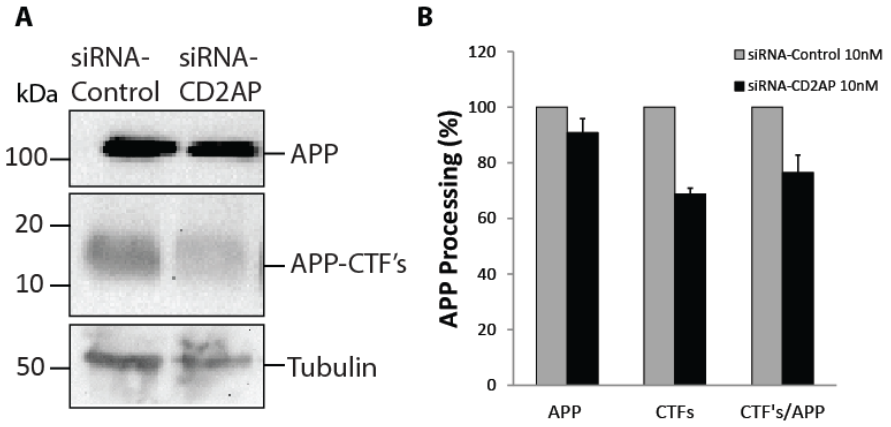


Figure 7 – CD2AP knockdown affects APP degradation in primary neurons.
Primary neurons (9 DIV) were transfected, as described in *Methods*, with siRNA-CD2AP or siRNA-control at 10nM. **(A)** Western blot analysis of PN with anti-APP (Y188) and α-tubulin. **(B)** Densitometric analysis of WB of PN, normalized by total α-tubulin. PN treated with siRNA-CD2AP reveal APP level of 90.9 ±5 % and APP CTFs of 69.9 ±2 % compared to PN treated with siRNA-control. The ratio of CTFs/APP was 76.5 ±6.3% in PN treated with siRNA-CD2AP compared with PN treated with siRNA-control. The results are shown as percentage of the levels measured in PN treated with siRNA-control. (n=2)

CD2AP effects on APP Pathway

It was necessary define if CD2AP could change the intracellular trafficking pathway of APP and BACE1, to clearly understand the alterations observed in APP processing.

CD2AP role in the cellular mechanisms is far from being fully well-known. However the studies on CD2AP refer the involvement in the sorting and maturation of early endosomes to late endosomes in association with rab4 ([29]). Since APP processing occurs in endosomes we investigated if CD2AP localizes to endosomes with APP. We conducted an experiment with N2a cells transfected with GFP-CD2AP or Myc-CD2AP, after 24h cells were either incubated with Alexa transferrin-647 for 3 min and fixed to label EE or were fixed and immunolabeled with anti-Transferrin Receptor for RE, Cathepsin D for LE/LY, GM130 to label the cis-Golgi and (Fig. 8).

CD2AP partially colocalized with EE (Fig 8.A-D) and RE (Fig 8.E-H). The staining for Cathepsin D was inconclusive. Thus the possible localization of CD2AP to LE/LY needs

to be verified (Fig 8.I-L). CD2AP did not colocalize with GM130 but the two signal remains near. Reveling, the CD2AP produced in excess accumulates in the Golgi.

We prove that CD2AP at steady-state is present in some way with EE and RE, the CD2AP did not change the endosomes distribution.

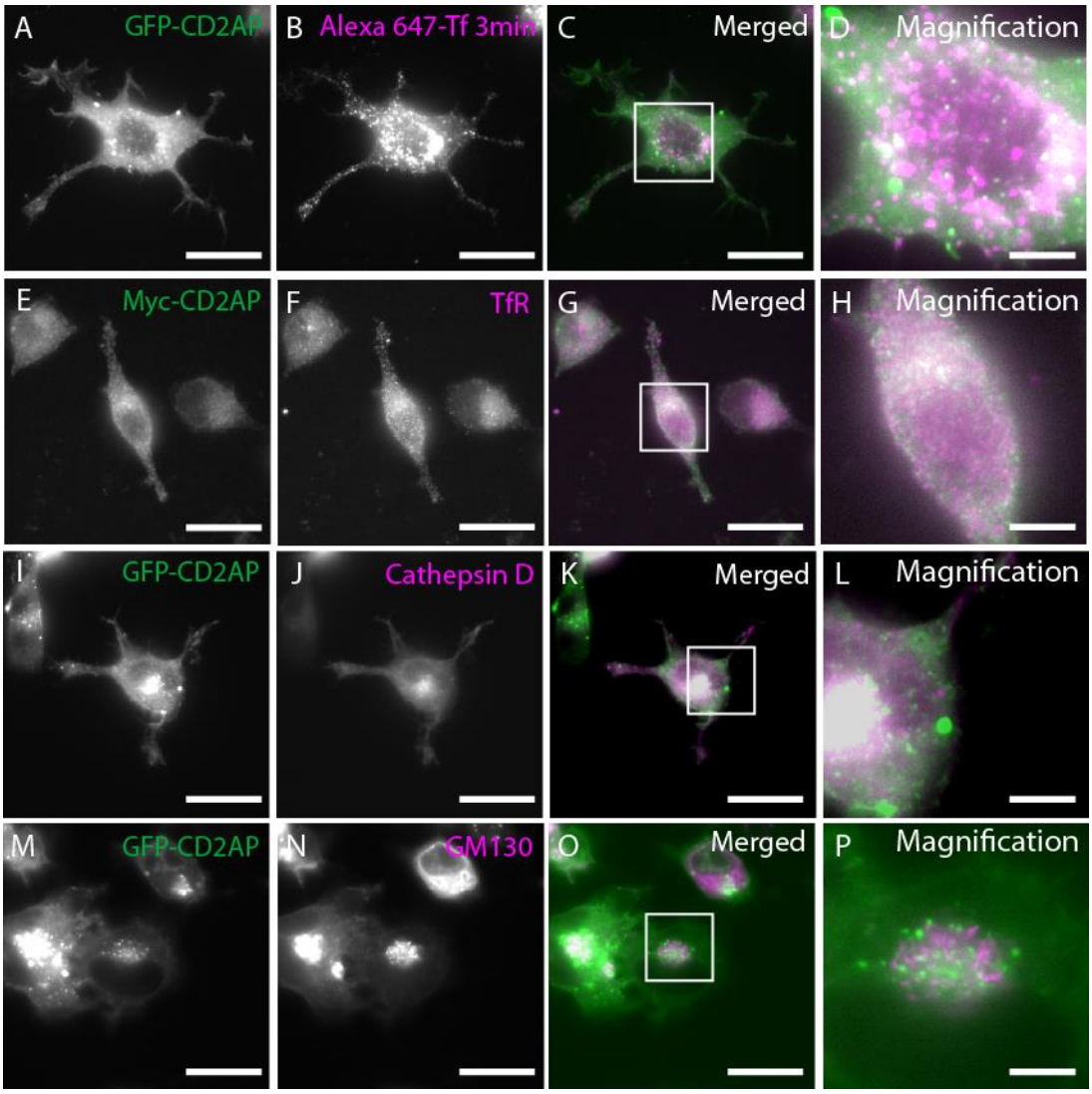
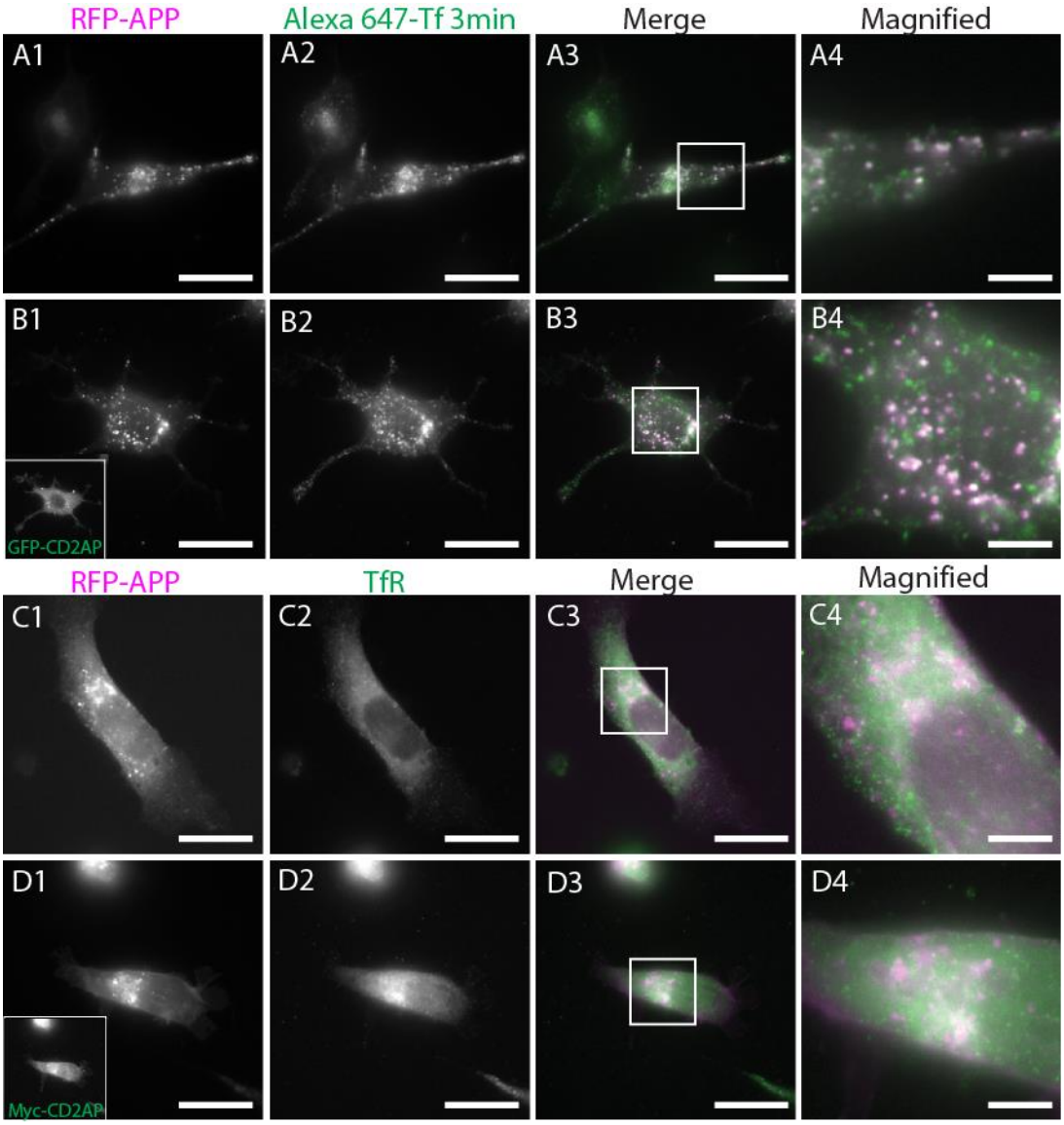


Figure 8 – Distribution of CD2AP for endosomes.
N2a cells were transfected with GFP-CD2AP, as described in *Methods*. (**A-D**) Cells labeled for early endosomes by 3 min pulse of transferrin (Alexa Fluor 647), (**E-H**) recycling endosomes, (**I-L**) late endosomes (**M-P**) cis-golgi. Scale bar 20µm.

N2a cells transfected with RFP-APP alone or with RFP-APP plus GFP-CD2AP or Myc-CD2AP were after 24h of expression incubated with Alexa transferrin-647 for 3 min and fixed to label EE and fixed (Fig. 9 A-B), or permeabilized and stained for; transferrin receptor (TfR) to recycling endosomes (RE, Fig. 8 C-D), Cathepsin D to label late

endosomes/lysosomes (LE/LY; Fig. 8 E-F), GM130 to label the Golgi apparatus (FIG. 8 G-H). We investigated if the overexpression of CD2AP altered the colocalization of RFP-APP with each organelle marker. Analyzing IF images we saw that APP colocalizes with EE, CD2AP expression did not show a visible effect. The marker for RE did not colocalize with APP independently of CD2AP expression. Cathepsin D staining was not good enough to take conclusions. In N2a cells labeled for Golgi, the APP was found next to GM130 staining, consistent with APP being concentrated more in the TGN than in the cis-Golgi, labeled by GM130, independently of CD2AP. The levels of APP present GFP-expressing N2a cells and CD2AP-expressing N2a cells was not significantly different. The results show that endogenous APP colocalizes with CD2AP on PM region, in other regions of cell, we did not see a direct colocalization between APP and CD2AP (Fig 9.I). In summary the results confirmed that APP is endocytosed from the PM into EE.



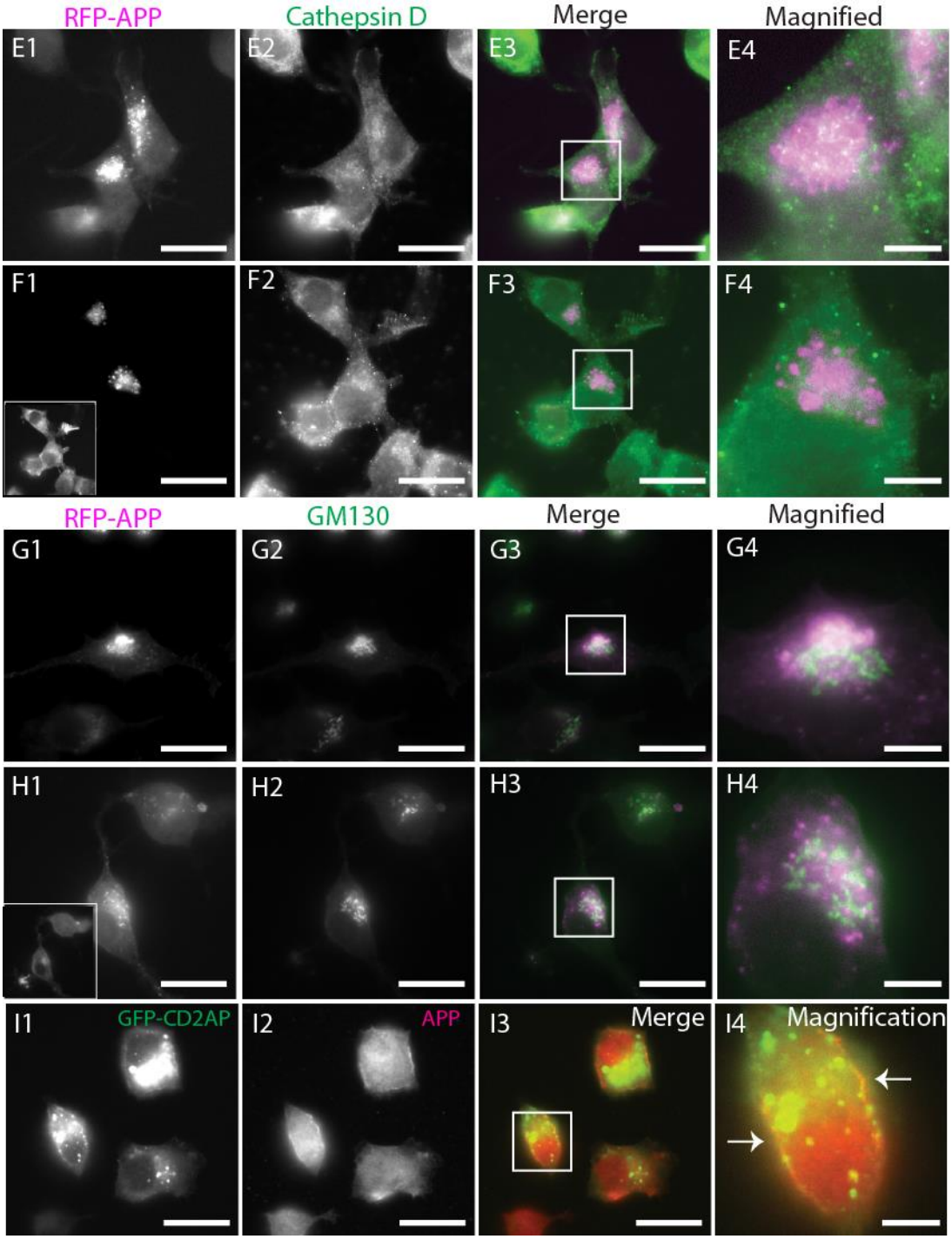


Figure 9 – CD2AP overexpression did not affect RFP-APP distribution in N2a cells.

N2a cells were transfected, as described in *Methods* with RFP-APP plus CD2AP tagged by GFP or Myc, in control situation the cells were transfected only with RFP-APP. (**A-B**) N2a cells were incubated for 3 min at RT with transferrin (Alexa Fluor 647-transferrin) to label the EE. (**C-D**) Co-staining with anti-myc and anti-transferrin receptor to label the RE. (**E-F**) Staining with anti-cathepsin D to label the LY. (**G-H**) Staining with anti-GM130 to label the cis-golgi. (**I**) N2a cells were transfected with GFP-CD2AP and incubated with anti-APP (22c11) arrows indicating colocalization on PM. Scale bar 20µm

Next we started to investigate the effect of CD2AP knockdown on endogenous APP. Thus far we colocalized endogenous APP with Lamp1, a marker of late-endosomes/lysososmes in GFP- or CD2AP-GFP expressing n2a cells (Fig. 9). In our preliminary we observed that endogenous APP is carried by endosomes positives for Lamp1 and that the number of endosomes carrying endogenous APP increased by 74% in N2a cells KD for CD2AP. These results are consistent with the pathway described in previous study where Gauthier et al was observed when CD2AP is KD ([35]), with an increase of APP in endosomes Lamp1 positives when CD2AP was KD. The differences between the decrease of cargo in LE described by Gauthier et al and the increase of APP in LE is dependent of the characteristics of proteins processing.

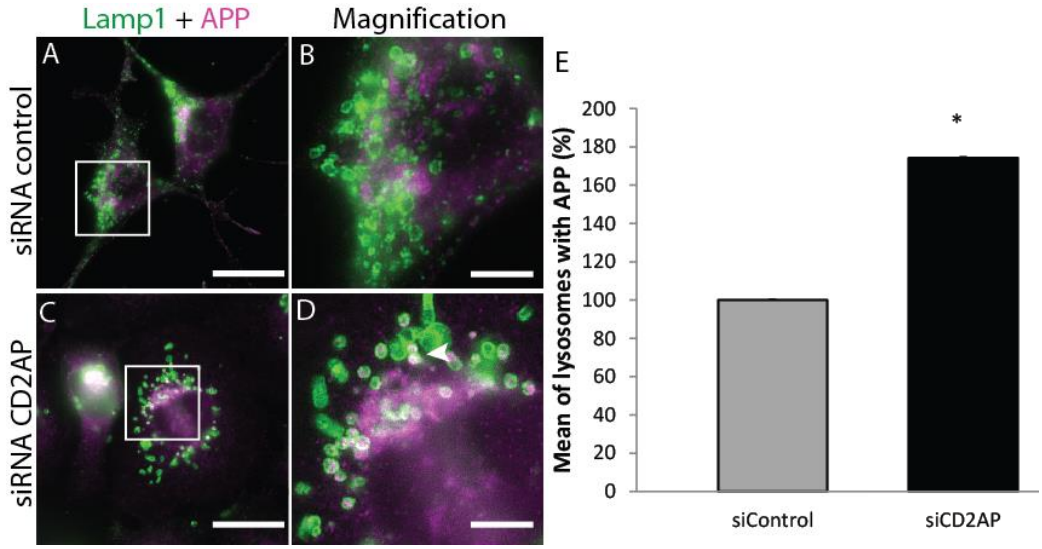


Figure 10 – CD2AP knockdown increase APP in Lysosomes.

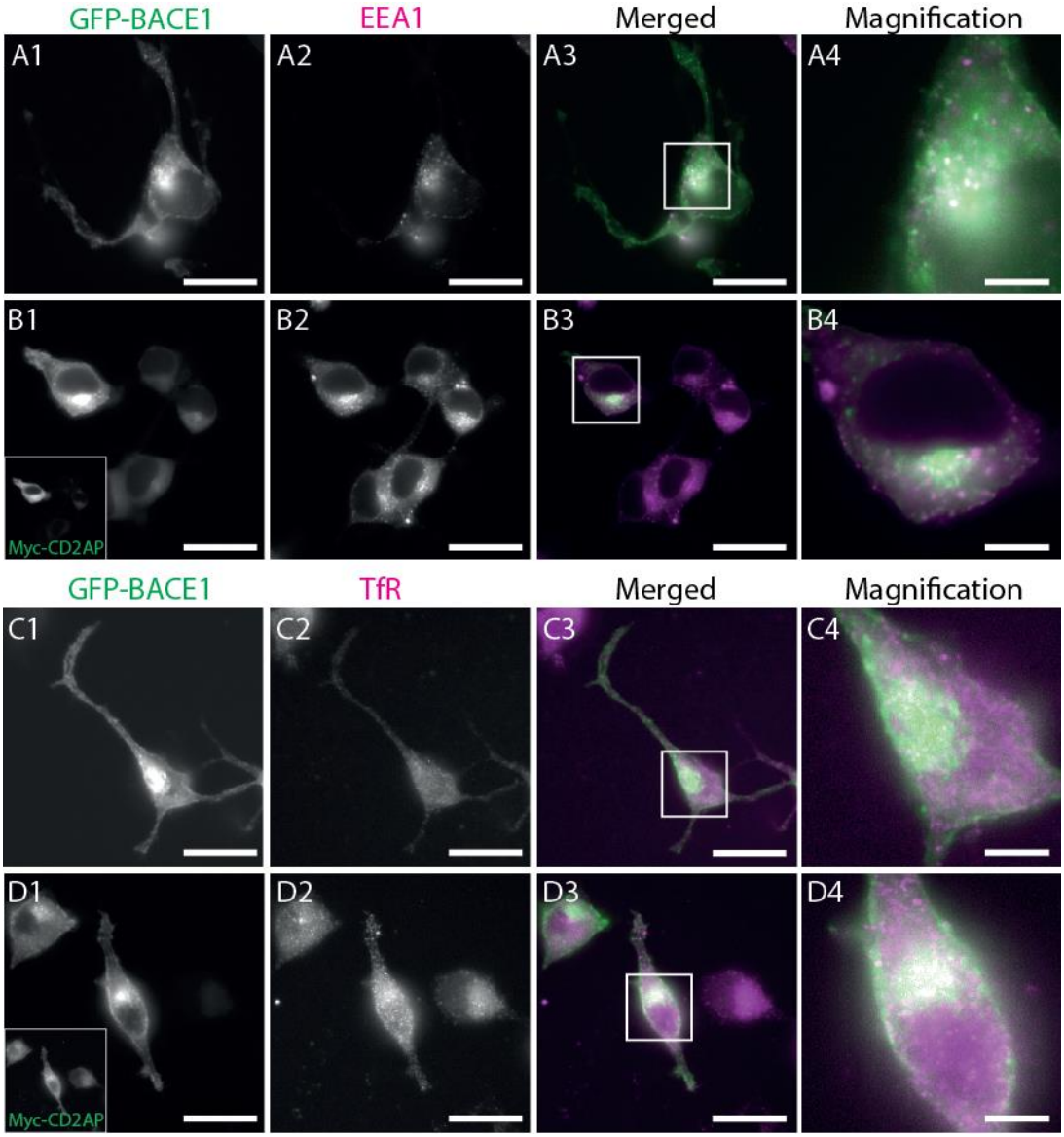
N2a cells were treated, as described in *Methods* with siRNA-control and siRNA-CD2AP at 10nM. (**A, C**) Cells stained for co-staining with anti-APP (22c11) anti-Lamp1, marker for LE; (**B, D**) Magnification from white box in A and C. (**E**) Analyze the number of endosomes positive for Lamp1 carrying APP/CTFs (174,2 ± 5.0%; p <0.01 n=1, N=95). The results are shown as percentage of the number measured in N2a treated with siRNA-control. Scale bar 20µm.

CD2AP effects on BACE1 pathway

Next we analyzed if CD2AP affects the cellular distribution of BACE1 in N2a cells. N2a cells overexpressing GFP-BACE1 alone or plus Myc-CD2AP were fixed 24h after transfection and IF was performed antibodies against markers for early endosomes, EEA1, late endosomes, Cathepsin D and recycling endosomes, TfR. Studies on the distribution of BACE1 are not consensual ([10]). We found that BACE1 colocalized partially with EEA1 (Fig 11.A-B) and TfR (FIG 11.C-D). The distribution of BACE1 remains unchanged when

CD2AP was overexpressed. BACE1 did not show colocalization with LE. These results experiment confirm that BACE1 is transported from PM to RE via EE independently of CD2AP overexpression. This experiment also revealed colocalization between CD2AP and BACE1 (Fig 11.G).

In summary the results of BACE1 and APP distribution confirm that, BACE1 can meet APP in PM or EE; CD2AP is present in endosomes carrying APP and BACE1; cells CD2AP depleted the APP level in endosomes positives for Lamp1 decrease. Together this data provide new clues to the role of CD2AP in cellular traffic.



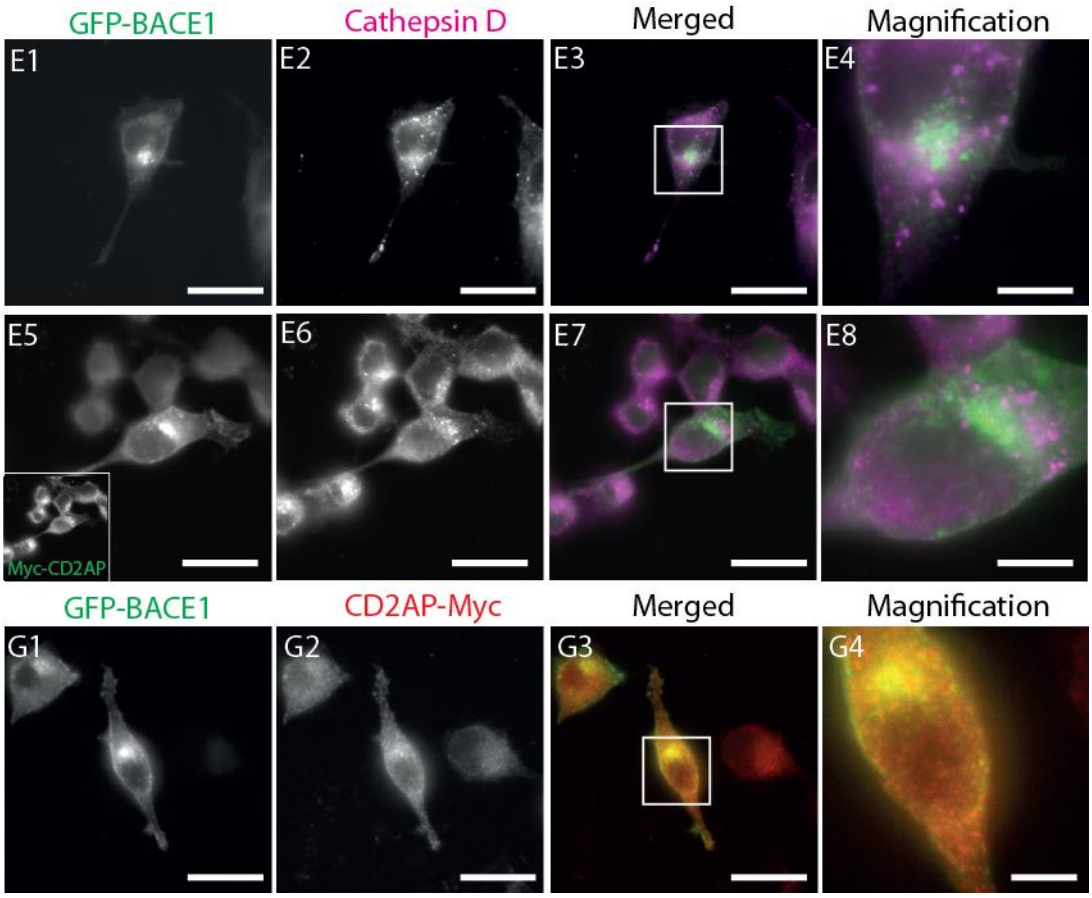


Figure 11 – CD2AP overexpression did not affect BACE1 distribution in N2A cells.

N2a cells were transfected, as described in *Methods* with GFP-BACE1 plus CD2AP tagged by Myc, in control situation the cells were transfected only with GFP-BACE1. Cells transfected with myc tag were labeled with anti-myc to detect CD2AP. (**A-B**) N2a cells were incubated with EEA1 to label the EE, cell marker to EE. (**C-D**) Cells were stained with anti-transferrin receptor marker to RE. (**E-F**) Cells were stained with anti-cathepsin D marker to LY. (**G**) N2a cells indicating the colocalization between Myc-CD2AP and GFP-BACE1 signal. Scale bar 20µm.

CD2AP effects on the Actin and cell morphology

Was mentioned in the literature, CD2AP is involved in cell polarization and in formation of F-actin structures and this structures are associated to EE movement away from membrane to LE ([42];[35]). To study the CD2AP KD effect on F-actin and shape of our cell models, we transfected the PN and N2 cells with siRNA-CD2AP and siRNA-control at 10nM. After 72h of transfection, the cells were labeled with phaloidin a fungi toxin that stain F-actin, the conformation of cells was altered. Cells expressing CD2AP have long processes (Fig 13.A-C) by other side the CD2AP KD cells show several spicules with short processes (Fig 13.B-D). Measuring the signal of phaloidin we found an increase of 875% in N2a cells and

134.3% in PN CD2AP depleted.

Our result proved the role of CD2AP in regulation of F-actin in neurons and N2a. Interesting, we didn't find a perfect colocolization between CD2AP and F-actin, confirming the results from Lynch et all 2007, that suggest the interaction endophilin-CD2AP-cortactin-Arp2/3-F-actin, on the endocytosis of proteins from membrane.

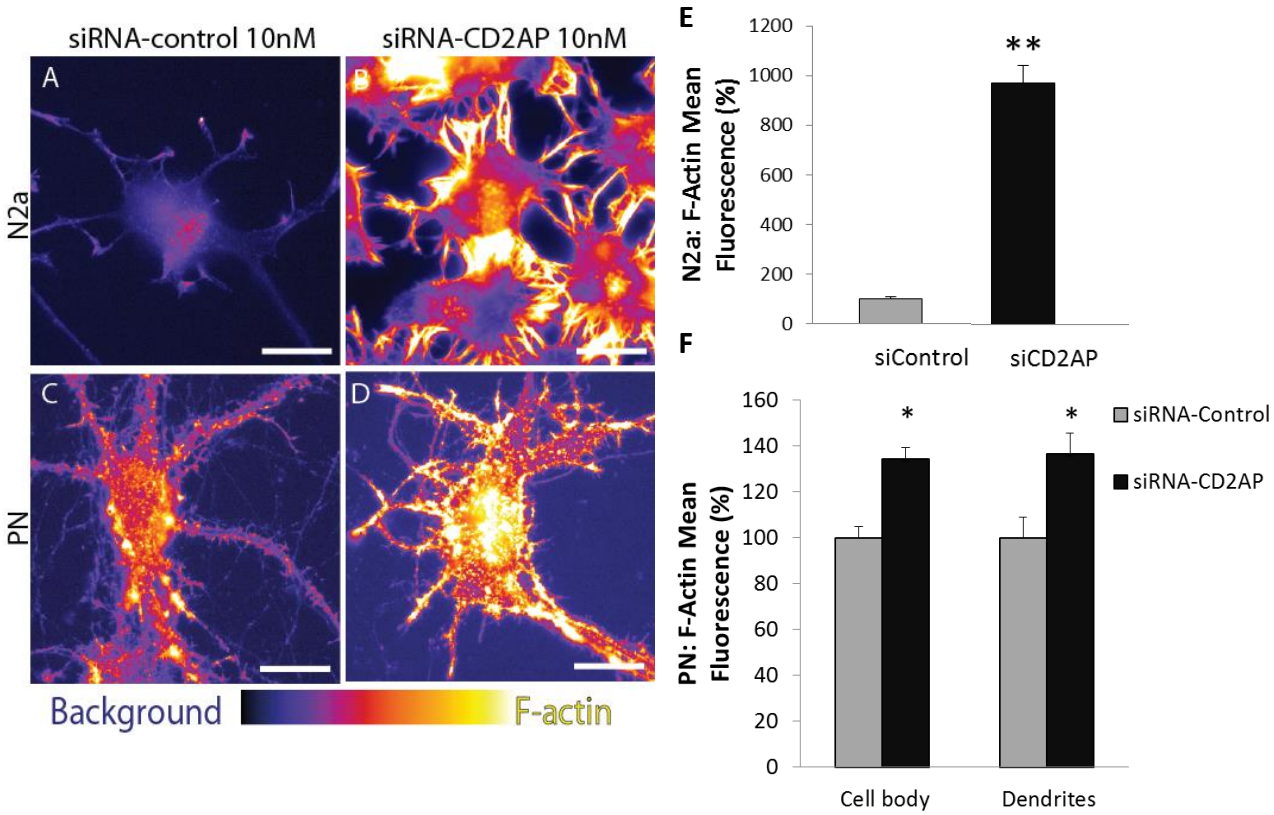


Figure 13 – CD2AP knockdown increase F-actin level.

N2a cell and Primary neurons were treated as described in *Methods*, with siRNA-CD2AP or siRNA control at 10nM. (**A-B**) N2a cells (**C-D**) PN were incubated with phalloidin a cell marker to F-actin, the images show the effects CD2AP in cell shape and in f-actin intensity. (**E**) Quantification of f-actin level, siRNA-CD2AP has 975 ±70.6% F-actin fluorescence (P <0.01; n=3; N=90) for N2a, (**F**) PN has 134.3 ±9.0% in cell body and 136.3 ±9.3% in dendrites when compared with respective controls (P <0.01 ; n=3; N=27). Scale bar 20µm.

Discussion

Genome-wide association studies show CD2AP increases the risk for AD in patients. While the CD2AP role is known in podocytes, and diseases such as kidney failure, in neurons the CD2AP role is unknown. Our laboratory has as one of its objectives, to study some of the risk factors for AD. As part of the completion for my dissertation, I was in charge of studying the role of CD2AP as a risk factor for AD to the molecular mechanisms that can lead to AD.

We started the study confirming the presence of CD2AP in mouse primary neurons, our cellular model. CD2AP was present in PN and it had a broad distribution throughout the cell with a higher concentration near the PM of cell body and a weaker expression in axons. CD2AP higher concentration in PM of neurons suggests that CD2AP could function in PN during receptor endocytosis. CD2AP in N2a cells was distributed throughout the cell with a higher concentration in PR. In HeLa cells we saw CD2AP scattered throughout the cell and at the PM but in some cells we saw puncta of higher CD2AP concentration in membrane extensions connecting two cells consistent with the localization of CD2AP to the midbody as described by N. Gauthier ([35]), due to the role of CD2AP during cytokinesis.

The initial accumulation of Aβ42 is a landmark for an early stage of the AD. To demonstrate that CD2AP can affect neurons leading an increase Aβ42 levels, we interfered with the regular expression of CD2AP. The experiments of CD2AP knockdown and overexpression in PN and N2a cells showed a significant increase on cell-associated Aβ42 mostly in dendrites. This increase in Aβ42 accumulation, suggests that CD2AP regulates in some way the pathway of Aβ42 production or of Aβ42 degradation. Until now the studies only described a genetic association between CD2AP and AD, but our results suggest for the first time that an alteration on expression of CD2AP in neurons lead to Aβ42 accumulation and thus contribute to AD development.

The next step in the study was to understand how CD2AP regulates the amyloidogenic pathway. Our analysis of APP processing indicated that both CD2AP overexpression and knockdown significantly decreased of the levels of APP C-terminal fragments and consequently a decrease in the CTFs/APP ratio. β-APP CTF is product of BACE1 cleavage and substrate for γ-secretase. The decrease in APP CTFs could be either due to reduced β-secretase processing of APP or increased γ-secretase processing of beta-APP CTFs. Since Aβ42 accumulates when CD2AP levels are altered it is likely that CD2AP is regulating the γ-

secretase and not β-secretase activity. These results confirm that CD2AP has role in amyloidogenic pathway with the consequent increase of Aβ42

To try to understand how CD2AP was altering APP processing we investigated CD2AP, APP and BACE1 localization along the endocytic pathway. At the plasma membrane we observed CD2AP colocalization with APP but not with BACE1. Indeed CD2AP has been described to function at the PM in receptor mediated endocytosis in a complex with Cbl and endophilin (ref). After internalization we could detect CD2AP in endosomes, especially in early endosomes that also contain APP and BACE1. Since CD2AP overexpression did not alter the presence of APP or BACE1 in early endosomes it suggest that CD2AP is not regulating the internalization of APP and BACE1, although more experiments will be needed to confirm this. For the rest of the endocytic pathway it was not obvious that CD2AP shared other compartments with APP or BACE1 or if it altered their distribution to recycling endosomes, late endosomes/lysosomes or the Golgi

Although overexpression of CD2AP did not alter significantly the distribution of APP and BACE1 in N2a cells down-regulated for CD2AP expression increases the signal of anti-APP (Y188) in LE/LY, sustained the hypothesis that CD2AP functions later in the endocytic pathway probably involved in endosomal maturation and sorting of proteins to the degradation pathway ([43]). Because the Y188 is an antibody for C-terminal of APP, the signal can correspond to APP full length or to APP CTF. , the increased presence of APP CTFs in late endosomes indicates that this is the site γ-secretase is active supported by the fact that endosomes positives for Lamp1 have an acidic pH (ref), . The results suggest that the regular expression of CD2AP controls the trafficking of APP or beta-CTF to LE/LY avoiding a more efficient cleavage by γ-secretase.

Finally we started to investigate how CD2AP is regulating Aβ42 accumulation and APP trafficking. Interestingly we found that both in N2a cells and primary neurons, that CD2AP has an important role in F-actin regulation. Cells depleted of CD2AP show a higher increase in F-actin level and stress fibers with a disorganizations F-actin positive membrane extensions (likely filopodia) and the disappearance of cellular processes. Indeed, CD2AP has been described to bridge actin filaments to endosomes and besides interacting with cortactin (actin regulator) can directly control actin polymerization since it has capping activity ([35,42,44])

In conclusion our results show that CD2AP plays a key role in cellular organization, regulating the actin filaments. The deregulation of CD2AP expression leads to increased levels of Aβ42 mostly in dendrites, increasing the risk of developing the AD. We propose in the light of our results and the existing literature, that CD2AP is involved in and the sorting of APP or β-CTFs from EE to LE/LY. More work will be necessary to confirm our conclusions and to understand the specific role of CD2AP on the development of Alzheimer disease.

Future perspectives

In this work, we prove that a deregulation on CD2AP expression, lead to an intracellular Aβ42 accumulation. We have provided some important clues about the effect of CD2AP in APP pathway, but we need more results to better understand the effect of CD2AP in endosomes, and biochemical assays, to know as CD2AP binds and interact with endosomes. The next step in the study of CD2AP as risk factor for AD, is verify if CD2AP is involved in Aβ42 degradation pathway. Studding the CD2AP role on traffic of Aβ degradation enzymes and the level of Aβ42 degraded, to study we will change the level of CD2AP expression in neurons by DNA transfection or RNA interference. The second phase will be identify how the altered expression of CD2AP may affect synapses and recap synaptic dysfunction present in Alzheimer's disease. Also changing the level of CD2AP expression, measuring the effect on the levels of Aβ42 and different synaptic markers, including PSD-95 (dendritic spines) and GluA1 / 2 (glutamate receptors).

In summary, it is expected to discover a novel mechanism by which CD2AP (onset risk factor for AD) causes the accumulation of Aβ42 and lead to a synaptic degeneration. This new mechanism could, in parallel to the change of APP trafficking, contribute to the accumulation of Aβ42 and lead to the onset of AD. This new knowledge will bring new and effective therapeutic approaches, in the treatment of LOAD. Opening new ways in research areas such as gene therapy directed to CD2AP.

References

1. Kidd, P. M. Alzheimer's disease, amnestic mild cognitive impairment, and age-associated memory impairment: current understanding and progress toward integrative prevention. *Altern. Med. Rev. J. Clin. Ther.* **13,** 85–115 (2008).
2. Bettens, K., Sleegers, K. & Van Broeckhoven, C. Current status on Alzheimer disease molecular genetics: from past, to present, to future. *Hum. Mol. Genet.* **19,** R4–R11 (2010).
3. Takahashi, R. H. Oligomerization of Alzheimer's -Amyloid within Processes and Synapses of Cultured Neurons and Brain. *J. Neurosci.* **24,** 3592–3599 (2004).
4. Gouras, G. K. Convergence of Synapses, Endosomes, and Prions in the Biology of Neurodegenerative Diseases. *Int. J. Cell Biol.* **2013,** (2013).
5. Gouras, G. K., Almeida, C. G. & Takahashi, R. H. Intraneuronal Aβ accumulation and origin of plaques in Alzheimer's disease. *Neurobiol. Aging* **26,** 1235–1244 (2005).
6. Almeida, C. G. *et al.* Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. *Neurobiol. Dis.* **20,** 187–198 (2005).
7. Neefjes, J. & van der Kant, R. Stuck in traffic: an emerging theme in diseases of the nervous system. *Trends Neurosci.* **37,** 66–76 (2014).
8. Wilson, J. H. & Hunt. *Molecular biology of the cell, 5th edition.* (Garland Science, 2008).
9. Zhang, Y., Thompson, R., Zhang, H. & Xu, H. APP processing in Alzheimer's disease. *Mol. Brain* **4,** 3 (2011).
10. Chia, P. Z. C. *et al.* Intracellular itinerary of internalised β-secretase, BACE1, and its potential impact on β-amyloid peptide biogenesis. *Traffic Cph. Den.* **14,** 997–1013 (2013).
11. Zhang, X. & Song, W. The role of APP and BACE1 trafficking in APP processing and amyloid-? generation. *Alzheimers Res. Ther.* **5,** 46 (2013).
12. Huse, J. T. beta -Secretase Processing in the Trans-Golgi Network Preferentially Generates Truncated Amyloid Species That Accumulate in Alzheimer's Disease Brain. *J. Biol. Chem.* **277,** 16278–16284 (2002).

13. Jacobsen, J. S., Reinhart, P. & Pangalos, M. N. Current Concepts in Therapeutic Strategies Targeting Cognitive Decline and Disease Modification in Alzheimer's Disease. *NeuroRx* **2,** 612–626 (2005).
14. Sannerud, R. *et al.* ADP ribosylation factor 6 (ARF6) controls amyloid precursor protein (APP) processing by mediating the endosomal sorting of BACE1. *Proc. Natl. Acad. Sci. U. S. A.* **108,** E559–E568 (2011).
15. Prabhu, Y. *et al.* Adaptor protein 2-mediated endocytosis of the ?-secretase BACE1 is dispensable for amyloid precursor protein processing. *Mol. Biol. Cell* **23,** 2339–2351 (2012).
16. He, X., Li, F., Chang, W.-P. & Tang, J. GGA Proteins Mediate the Recycling Pathway of Memapsin 2 (BACE). *J. Biol. Chem.* **280,** 11696–11703 (2005).
17. Wahle, T. *et al.* GGA proteins regulate retrograde transport of BACE1 from endosomes to the trans-Golgi network. *Mol. Cell. Neurosci.* **29,** 453–461 (2005).
18. Huse, J. T., Pijak, D. S., Leslie, G. J., Lee, V. M.-Y. & Doms, R. W. Maturation and Endosomal Targeting of -Site Amyloid Precursor Protein-cleaving Enzyme: THE ALZHEIMER'S DISEASE -SECRETASE. *J. Biol. Chem.* **275,** 33729–33737 (2000).
19. Koo, E. H., Squazzo, S. L., Selkoe, D. J. & Koo, C. H. Trafficking of cell-surface amyloid beta-protein precursor. I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody. *J. Cell Sci.* **109 (Pt 5),** 991–998 (1996).
20. Page, R. M. *et al.* Loss of PAFAH1B2 Reduces Amyloid- Generation by Promoting the Degradation of Amyloid Precursor Protein C-Terminal Fragments. *J. Neurosci.* **32,** 18204–18214 (2012).
21. Zou, Z., Liu, C., Che, C. & Huang, H. Clinical Genetics of Alzheimer's Disease. *BioMed Res. Int.* **2014,** (2014).
22. Blennow, K., de Leon, M. J. & Zetterberg, H. Alzheimer's disease. *Lancet* **368,** 387–403 (2006).
23. Isik, A. T. Late onset Alzheimer's disease in older people. *Clin. Interv. Aging* **5,** 307–311 (2010).
24. Hollingworth, P. *et al.* Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nat. Genet.* **43,** 429–435 (2011).

25. Naj, A. C. *et al.* Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat. Genet.* **43,** 436–441 (2011).
26. Karch, C. M. *et al.* Expression of Novel Alzheimer's Disease Risk Genes in Control and Alzheimer's Disease Brains. *PLoS ONE* **7,** e50976 (2012).
27. Rosenthal, S. L. & Kamboh, M. I. Late-Onset Alzheimer's Disease Genes and the Potentially Implicated Pathways. *Curr. Genet. Med. Rep.* **2,** 85–101 (2014).
28. Dustin, M. L. *et al.* A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* **94,** 667–677 (1998).
29. Cormont, M. *et al.* CD2AP/CMS Regulates Endosome Morphology and Traffic to the Degradative Pathway Through its Interaction with Rab4 and c-Cbl. *Traffic* **4,** 97–112 (2003).
30. Monzo, P. *et al.* Clues to CD2-associated protein involvement in cytokinesis. *Mol. Biol. Cell* **16,** 2891–2902 (2005).
31. Kirsch, K. H., Georgescu, M.-M., Ishimaru, S. & Hanafusa, H. CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc. Natl. Acad. Sci.* **96,** 6211–6216 (1999).
32. Dikic, I. CIN85/CMS family of adaptor molecules. *FEBS Lett.* **529,** 110–115 (2002).
33. Lehtonen, S., Zhao, F. & Lehtonen, E. CD2-associated protein directly interacts with the actin cytoskeleton. *AJP Ren. Physiol.* **283,** F734–F743 (2002).
34. Wolf, G. & Stahl, R. A. CD2-associated protein and glomerular disease. *The Lancet* **362,** 1746–1748 (2003).
35. Gauthier, N. C. *et al.* Early endosomes associated with dynamic F-actin structures are required for late trafficking of H. pylori VacA toxin. *J. Cell Biol.* **177,** 343–354 (2007).
36. Chen, H. *et al.* Analyzing 54,936 Samples Supports the Association Between CD2AP rs9349407 Polymorphism and Alzheimer's Disease Susceptibility. *Mol. Neurobiol.* 1–7 (2014). doi:10.1007/s12035-014-8834-2
37. Treusch, S. *et al.* Functional Links Between A? Toxicity, Endocytic Trafficking and Alzheimer's Disease Risk Factors in Yeast. *Science* **334,** 1241–1245 (2011).

38. Shulman, J. M. *et al.* Functional screening in Drosophila identifies Alzheimer's disease susceptibility genes and implicates Tau-mediated mechanisms. *Hum. Mol. Genet.* ddt478 (2013).
39. Morgan, K. The three new pathways leading to Alzheimer's disease: Three new pathways leading to AD. *Neuropathol. Appl. Neurobiol.* **37,** 353–357 (2011).
40. Provost, P. Interpretation and applicability of microRNA data to the context of Alzheimer's and age-related diseases. *Aging* **2,** 166–169 (2010).
41. Szodorai, A. *et al.* APP anterograde transport requires Rab3A GTPase activity for assembly of the transport vesicle. *J. Neurosci. Off. J. Soc. Neurosci.* **29,** 14534–14544 (2009).
42. Lynch, D. K. *et al.* A Cortactin-CD2-associated Protein (CD2AP) Complex Provides a Novel Link between Epidermal Growth Factor Receptor Endocytosis and the Actin Cytoskeleton. *J. Biol. Chem.* **278,** 21805–21813 (2003).
43. Welsch, T. Association of CD2AP with dynamic actin on vesicles in podocytes. *AJP Ren. Physiol.* **289,** F1134–F1143 (2005).
44. Zhao, J. *et al.* CD2AP Links Cortactin and Capping Protein at the Cell Periphery To Facilitate Formation of Lamellipodia. *Mol. Cell. Biol.* **33,** 38–47 (2013).

S. DATA:

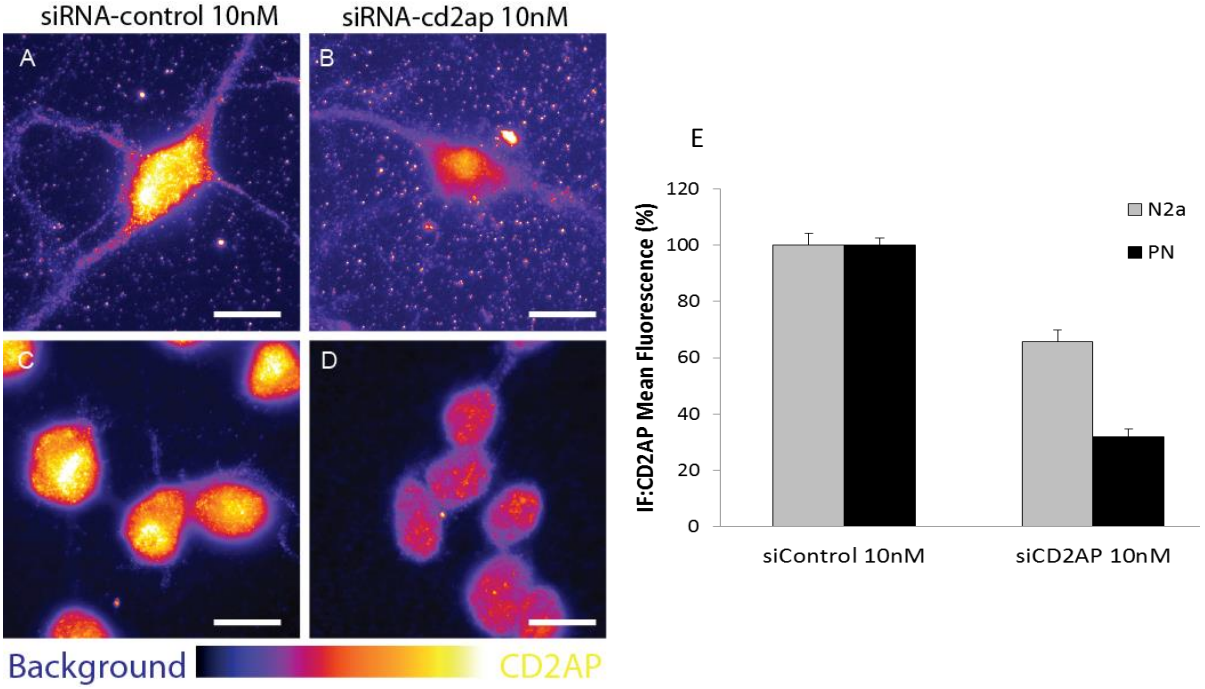


Figure S1 – siRNA-CD2AP decrease the level of endogenous CD2AP.
N2a cells were treated with CD2AP-siRNA or siRNA-control, using RNAiMax as described in *Methods*. **(A-B/C-D)** N2a cells where immunolabeled with anti-CD2AP, fluorescence intensity is pseudocolored in a blue-hot lut (dark blue is background, yeallow corresponds to the brightest signal)**. (E)** Quantification of mean CD2AP fluorescence in N2a cells treated with CD2AP siRNA was 65.6 ±2.7 compared to N2a cells treated with siRNA-control ($p < 0.001$; N=90 CD2AP siRNA cells; N= 72 SiRNA Control cells; n=4)

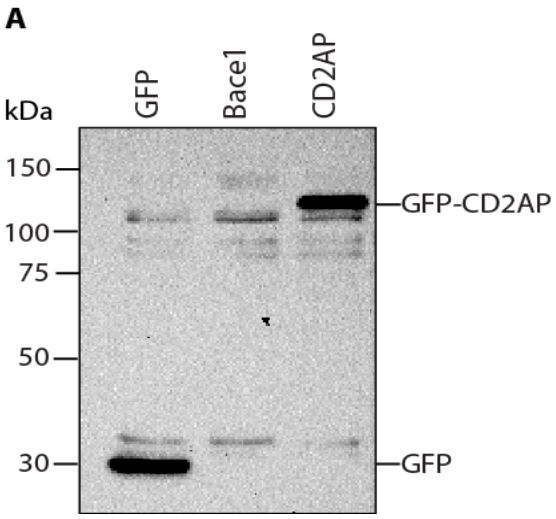


Figure S2 – WB confirmed the expression of CD2AP and BACE1 tagged with GFP.
(A) Western blot analysis of PN with anti-GFP.